

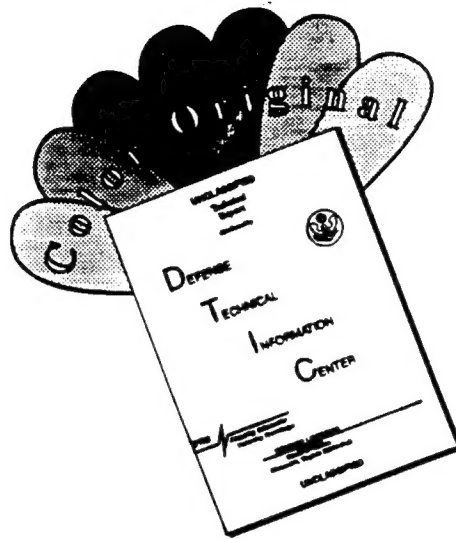
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**Regulation of Growth Zone and Resting Zone  
Chondrocytes by  
Bone Morphogenetic Protein - 2**

A Thesis

Presented to the Faculty of

The University of Texas Graduate School of Biomedical Sciences

at San Antonio

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Douglas M. Erickson, B.A., D.D.S.

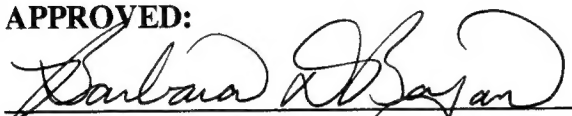
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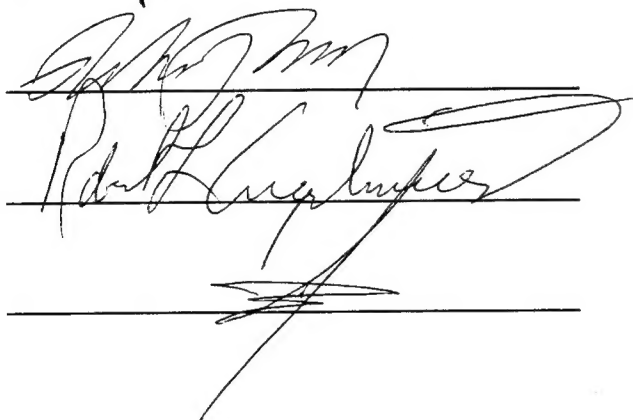
# REGULATION OF GROWTH ZONE AND RESTING ZONE CHONDROCYTES BY BONE MORPHOGENETIC PROTEIN - 2

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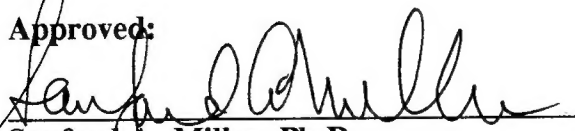




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## DEDICATION

This thesis is dedicated to my wife, Nancy, and my daughter, Sarah, whose unselfish sacrifices and understanding make it possible for any success that I may achieve. They have put up with a lot.

## ACKNOWLEDGMENTS

The author wishes to take this opportunity to thank the members of my research committee, the orthopaedic research laboratory, and the staff of the University Cooperative Research Center. Specifically, I would like to thank the chairperson on my research committee, Dr. Barbara Boyan for her commitment, encouragement, and her willingness to take a chance with a clinician that has not seen a scintillation vial in years. A special thanks goes to Dr. Zvi Schwartz whose continual drive, planning, and insight kept the momentum going. To Dr. David Dean I give thanks for his pragmatic and invaluable editorial prowess. To Dr. Steve Harris and his wife Marie who patiently lead me for many hours through the world of microbiology, I wish to extend a grateful thank you. A warm thank you to Bryan Brooks whose friendship and guidance kept me sane during my time in the lab and whom I could count on for moderating the influences that be. Appreciation is also extended to the entire laboratory staff, especially Monica Luna, Stephanie Scheele, and Ms. Sandy Messier for dedication and willingness to spend time after hours to get the job done. Finally, I would like to thank Drs. Robert Cronin and Robert Engelmeier for giving me the opportunity to make all of this possible.

## Regulation of Growth Zone and Resting Zone Chondrocytes by Bone Morphogenetic Protein - 2

Douglas M. Erickson, D.D.S.

The University of Texas Graduate School of Biomedical Sciences  
at San Antonio

Supervising Professor: Barbara D. Boyan, Ph.D.

Bone morphogenetic protein (BMP) plays a major role in endochondral bone formation in vivo. However, the mechanism of action of this growth factor is not clear. This study examined the effect of recombinant human BMP-2 (rhBMP-2) on chondrocyte differentiation, metabolism, and whether this effect depends on cell maturation. [ $^3\text{H}$ ]-Thymidine incorporation (DNA synthesis), [ $^3\text{H}$ ]-uridine incorporation (RNA synthesis), alkaline phosphatase specific activity, and [ $^3\text{H}$ ]-proline incorporation into collagenase digestible protein (CDP) or non-collagenase digestible protein (NCP) were measured. Cartilage cells were obtained from both growth zone (GC) and the less mature resting zone (RC) of rat costochondral cartilage. Confluent, fourth passage cells were cultured for 24-48 hours in DMEM (Gibco, Grand Island, N.Y.) plus 10% FBS (Gibco) containing 50ug/mL ascorbic acid and 0.05-100ng/mL rhBMP-2. rhBMP-2 had no effect on [ $^3\text{H}$ ]-thymidine incorporation by growth zone cells at

either 24 or 48 h, but stimulated DNA synthesis in resting zone cells (12.5-100ng/mL). [3H]-Uridine incorporation was increased in growth zone cells at 48 hours (50-100ng/mL), but not in resting zone cells; at 48 hours, both cell types had increased [3H]-uridine incorporation (25-100ng/mL). Alkaline phosphatase specific activity was increased in growth zone cells at 24 and 48 hours (12.5-100ng/mL). rhBMP-2 caused a biphasic stimulation of alkaline phosphatase specific activity in resting zone cells that was significant at 0.2-0.8ng/mL and at 12.5-100ng/mL. rhBMP-2 stimulated CDP and NCP production in both cell types over a concentration range of 3.1-25ng/mL. Percent collagen production was unaffected in either cell type. These results indicate that rhBMP-2 regulates chondrocyte proliferation, differentiation, and metabolism; however, this effect depends on cell maturation and concentration of BMP-2. The results also suggest that new endochondral bone formation, induced by BMP, may occur through direct regulation of chondrocyte differentiation.



## TABLE OF CONTENTS

DEDICATION . . . . .	iii
ACKNOWLEDGMENTS . . . . .	iv
LIST OF FIGURES. . . . .	ix
INTRODUCTION . . . . .	1
LITERATURE REVIEW . . . . .	4
Endochondral Bone Growth . . . . .	4
Matrix Vesicles . . . . .	8
Alkaline phosphatase . . . . .	15
Phospholipase A <sub>2</sub> . . . . .	16
Associated cartilaginous matrix proteins . . . . .	17
Characteristics of BMP . . . . .	18
Functions of BMP. . . . .	19
Differentiation and Proliferation . . . . .	20
Clinical applications of BMP . . . . .	21
Long-bone and trephine non-union defects . . . . .	22
Dental Implications . . . . .	25
Bone and Cartilage Models . . . . .	25
METHODS AND MATERIALS . . . . .	26
Chondrocyte Culture . . . . .	26
Determination of Alkaline Phosphatase Specific Activity . . . . .	27
Alkaline Phosphatase . . . . .	27
Total Protein. . . . .	28
Determination of [ <sup>3</sup> H]-Thymidine Incorporation. . . . .	28
Determination of Collagenase Digestible and Non-Collagenase Digestible Protein Production. . . . .	29
Determination of [ <sup>3</sup> H]-Uridine Incorporation . . . . .	31
Statistical Analysis . . . . .	32
RESULTS . . . . .	33
Alkaline Phosphatase . . . . .	33
[ <sup>3</sup> H]-Thymidine Incorporation . . . . .	34
Collagenase Digestible and Non-collagenase Digestible Protein Stimulation. . . . .	34
[ <sup>3</sup> H]-Uridine Incorporation . . . . .	35

<b>DISCUSSION . . . . .</b>	<b>58</b>
<b>LITERATURE CITED . . . . .</b>	<b>64</b>
<b>VITA . . . . .</b>	<b>72</b>

## LIST OF FIGURES

<b>Figure 1:</b>	Anatomy of a long bone growth plate undergoing endochondral ossification. . . . .	5
<b>Figure 2:</b>	Matrix vesicle biogenesis and maturation by chondrocytes or osteoblasts. . . . .	10
<b>Figure 3:</b>	Anatomy of a Matrix Vesicle. Diagram shows structure and biochemistry of a matrix vesicle. . . . .	12
<b>Figure 4:</b>	The effect of varying amounts of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on alkaline phosphatase specific activity in cell layers isolated from growth zone chondrocyte cultures, after 24 or 48 hours of treatment. . . . .	36
<b>Figure 5:</b>	The effect of varying amounts of rhBMP-2 on alkaline phosphatase specific activity in cell layers isolated from growth zone chondrocyte cultures after 24 or 48 hours of treatment. The data are expressed as treatment/control ratios for 6 experiments. . . . .	38
<b>Figure 6:</b>	The effect of varying amounts of rhBMP-2 on alkaline phosphatase specific activity in cell layers isolated from resting zone chondrocyte cultures after 24 or 48 hours of treatment. . . . .	40
<b>Figure 7:</b>	The effect of varying amounts of rhBMP-2 on alkaline phosphatase specific activity in cell layers isolated from resting zone chondrocyte cultures after 24 or 48 hours of treatment. The data are expressed as treatment/control ratios for 6 experiments. . . . .	42
<b>Figure 8:</b>	The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated thymidine incorporation by quiescent growth zone (GC) and resting zone (RC) chondrocytes. . . . .	44
<b>Figure 9:</b>	The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated thymidine incorporation after 24 or 48 hours in confluent, non-quiescent fourth passage costochondral growth zone chondrocytes. . . . .	46
<b>Figure 10:</b>	The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated thymidine incorporation after 24 or 48 hours in confluent, non-quiescent fourth passage costochondral resting zone chondrocytes. . . . .	48
<b>Figure 11:</b>	The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on tritiated proline incorporation into collagenase digestible protein. Confluent, fourth passage costochondral growth zone (GC) and resting zone (RC) chondrocytes were examined. . .	50

**Figure 12:**

The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated proline incorporation into non-collagenase digestible protein. Confluent, fourth passage costochondral growth zone (GC) and resting zone (RC) chondrocytes were examined. . . 52

**Figure 13:**

The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated uridine incorporation by confluent, fourth passage costochondral resting zone chondrocytes. . . . . 54

**Figure 14:**

The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated uridine incorporation by confluent, fourth passage costochondral growth zone chondrocytes. . . . . 56

## **I. INTRODUCTION**

Prosthodontics is defined as "the science of and art of providing suitable substitutes for the coronal portions of teeth, or for lost or missing teeth and their associated parts (Stedman's Pocket Medical Dictionary. Williams and Wilkins, Baltimore MD 1987, p. 615.)." Prosthetic treatment success, however, depend on the placement of these replacement teeth on a solid bony foundation. Commonly, that bony foundation has been ravaged by disease and age making prosthodontic therapy less than optimum. The question becomes how to replace the missing bone to allow for an esthetic and functional success.

Oral surgeons, periodontists, and endodontists have been moderately successful in grafting iliac bone to an atrophied mandible, tenting a maxillary sinus to allow for the growth of bony support needed for implants, grafting to fill in periodontal bony defects, using allogeneic bone grafts to patch mandibular non-union bony defects from surgery or trauma, and obturating a blunderbuss apical tooth root foramen. Efforts have been underway to discover new ways of regenerating bone since the mid-1800's.

Marshall Urist, in the 1960's, discovered that demineralized bone could stimulate bone formation in soft tissue. He named the putative active agent "bone morphogenetic protein (BMP)." Since that time, Urist purified BMP from bone and showed through many different approaches that "BMP-induced bone formation is a reaction of mesenchymal type cells with embryonic potential for osteogenesis (Urist, 1989)."

Success has been achieved in augmenting and regenerating bone in both animal and

human models using BMP. Canalis, *et al.* in 1985 were able to implant a BMP laden implant into the femur of rabbits causing bone diameter to increase by 30% over the control.

Nakashima, *et al.* (1990) found they could induce the formation of tubular dentin, after osteodentin formation, in response to crude BMP placed in an amputated tooth pulp. BMP has also been used to regenerate bone in non-union long bone defects in animal and human models (Heckman, *et al.*, 1991; Wang, *et al.*, 1991; Schmitz, *et al.*, 1990; Urist, 1989; Johnson, *et al.*, 1988; Ferguson, *et al.*, 1987; Nilsson, *et al.*, 1986; Takagi and Urist 1982).

Initial cloning of BMP produced several recombinant forms: BMP-1, BMP-2a (now known as BMP-2), BMP-2b (now known as BMP-4), and BMP-3. All of these clones are independently capable of inducing the formation of cartilage *in vivo*. At present, seven distinct BMPs have been identified (Wozney, *et al.*, 1988).

Endochondral ossification is the primary mechanism for the formation of embryonic bone and post fetal long bone growth in humans. The process involves production and maintenance of a cartilaginous matrix by reserve zone chondrocytes. In response to one or more stimuli, these cells differentiate into growth cartilage cells which proliferate, hypertrophy, and calcify their matrix. This, in turn, serves as a framework for replacement by new bone. Fracture healing occurs by a process similar to the endochondral ossification found in normal bone growth and development (Ketenjian and Arsenis, 1975; Boskey *et al.*, 1980). In both instances, cartilage differentiation and mineralization of the cartilaginous matrix is an important prerequisite for bone formation.

While BMP is known to induce endochondral ossification when implanted heterotopically in mesenchymal tissues, its effects on cells in the chondrocyte lineage are

unknown. The purpose of this study was to examine the effects of rhBMP-2 on cultured chondrocytes, using a model with cartilage cells at two distinct stages of endochondral differentiation. Chondrocytes were cultured in the presence of rhBMP-2, and the effect of BMP on cell proliferation, cell differentiation, and protein synthesis was determined as a function of both BMP concentration and time of exposure. Cell proliferation was measured as a function of cell number and the incorporation of [ $^3\text{H}$ ]-thymidine. Alkaline phosphatase specific activity was measured as a marker of cell differentiation. Total protein synthesis was assessed as a function of [ $^3\text{H}$ ]-proline incorporation into collagenase digestible and non-collagenase digestible protein. Total RNA synthesis was measured by [ $^3\text{H}$ ]-uridine incorporation. We also hypothesize that regulation of these cells is maturation dependent. We believe that, in a dose dependent manner, rhBMP-2 will stimulate production of proteins specific to a maturing chondrocyte undergoing differentiation in long bone growth plate.

## **II. LITERATURE REVIEW**

### **A. Endochondral Bone Growth**

Endochondral ossification is the primary method of bone formation in humans. The process involves production and maintenance of a cartilaginous matrix by reserve zone chondrocytes. In response to one or more stimuli, these cells differentiate into growth cartilage cells which proliferate, hypertrophy, and calcify their matrix. This, in turn, serves as a framework for replacement by new bone and further mineralization. Bone wound healing occurs by a process similar to the endochondral ossification found in normal bone growth and development (Ketenjian and Arsenis, 1975; Boskey *et al.*, 1980). In both instances, cartilage differentiation and matrix mineralization are important prerequisites for bone formation.

During endochondral bone formation, cartilage growth plates can be divided into distinct anatomic zones: the resting or reserve zone, the proliferative cell zone, the hypertrophic cell zone, and the zone of calcification (Boskey, 1981; Buckwalter, *et al.*, 1986; Hunziker and Schenk, 1987)(see figure 1). In the resting zone, chondrocytes are distributed throughout the cartilage matrix in an apparently random fashion. It is in the proliferative zone that the chondrocytes begin to form vertical columns which continue to expand the plate interstitially until they enter the prehypertrophic zone. Matrix vesicles are produced by proliferating chondrocytes and are found in the longitudinal septa in the territorial matrix (Anderson, 1969; Boskey, 1981; Poole, *et al.*, 1989). Within the hypertrophic zone, cells polarize and enlarge volumetrically. Morphometrically there are fourfold and tenfold increases in the mean cellular height and volume, respectively

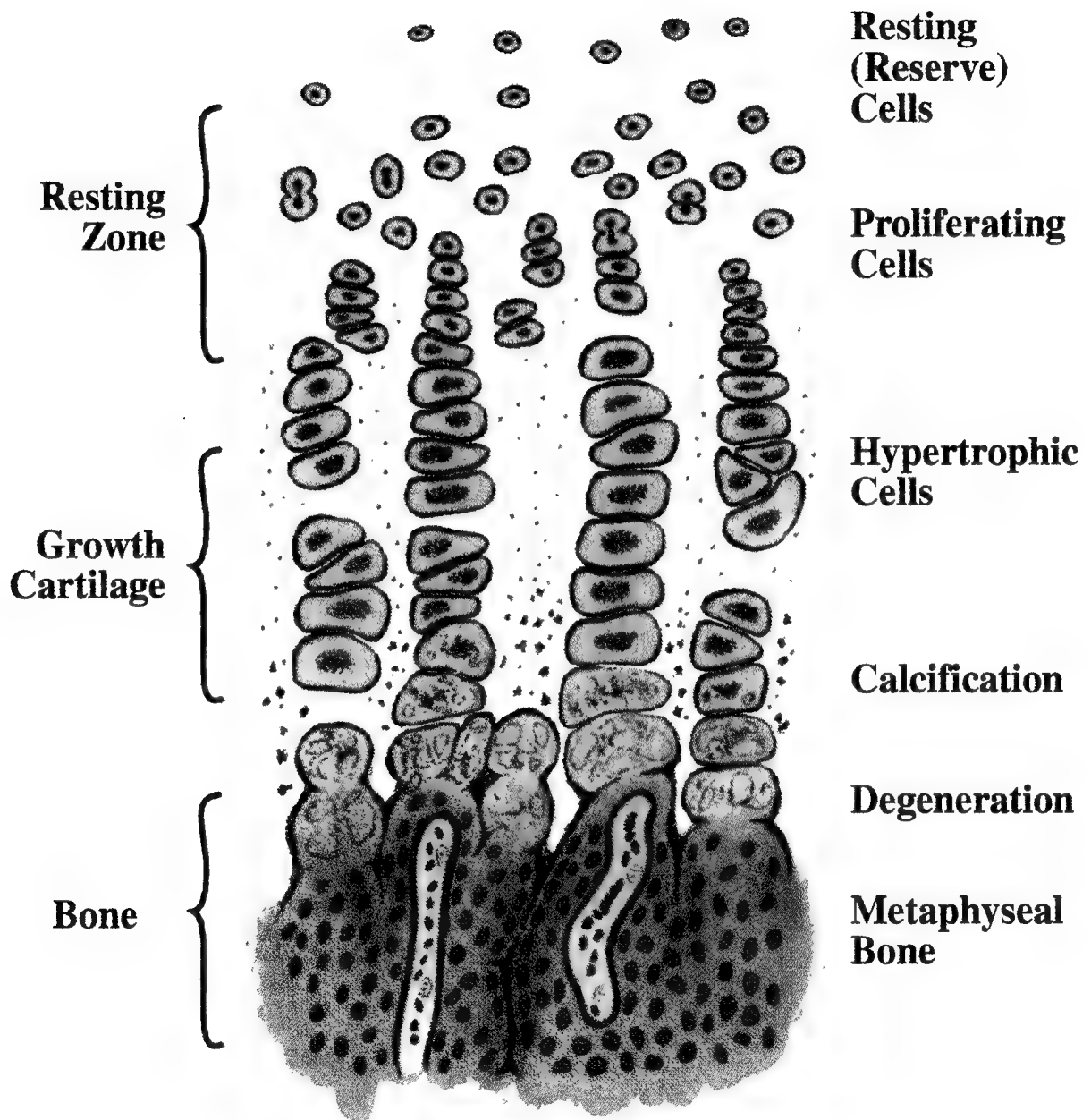


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**Figure 1:**

Anatomy of a long bone growth plate undergoing endochondral ossification. Illustration by D.B. Boyan.

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(Buckwalter, 1986; Hunziker and Schenk, 1987). Cytochemical studies have demonstrated that alkaline phosphatase levels increase as the cells approach the calcification front and that these levels are strongly related to the presence of extracellular vesicles (Matsuzawa and Anderson, 1971).

During the late stages of hypertrophy, calcification of the interterritorial matrix begins. The calcification is limited to the longitudinal septa between the columns of cells. "The pericellular and territorial matrix compartments within vertical and horizontal matrix septa compartments remain unmineralized and are resorbed at the vascular invasion front together with terminal chondrocytes (Hunziker and Shenk, 1987)." In the zone of calcification, the calcifying matrix serves as a lattice for the deposition of bone in the primary spongiosa. Here the chondrocytes vacuolate and may die. Using stereological estimators which describe cellular height, the rates of cellular turnover can be calculated. The rapid progression of the vascular invasion front was found to eliminate, for each column of cells, one chondrocyte every three hours; that is, eight cells a day (Hunziker and Shenk, 1987). During the first bone apposition-resorption formation sequence, the calcified cartilage matrix is partially resorbed by osteoclasts, leaving longitudinal septa of bone. These osteoblasts subsequently differentiate and form a layer of woven bone on top of the cartilaginous remains of the longitudinal septa. Lower in the growth plate another apposition-resorption sequence leads to the replacement of woven bone and cartilaginous remnants with lamellar bone, or secondary spongiosa.

Intracellularly, chondrocytes within the growth plate are seen to undergo several changes prior to the calcification of their matrix. Initially, a progressive accumulation of glycogen occurs with a sudden disappearance, coincident with the onset of calcification. In

addition there is a gradual increase in total lipid, a shift from neutral to acid glycosaminoglycan, a marked increase in intracellular alkaline phosphatase, an increase in the activity of mitochondrial enzymes, and a decrease in glycolytic enzyme activities. Structurally there is also an increase in the number of mitochondria, and a twofold to fivefold increase in the mean cellular surface area of rough endoplasmic reticulum, the Golgi membranes and the mean cellular mitochondrial volume (Ketenjian and Arsenis, 1975; Hunziker, *et al.*, 1987).

In summary, growth plate cartilage is a tissue composed of chondrocytes at several distinct stages of differentiation. These cells mature through an endochondral differentiation pathway in a tightly regulated fashion that culminates in calcification of the matrix (Boskey, 1981).

#### B. Matrix Vesicles

Extracellular vesicles found in the growth plate were originally described as "crystal ghosts" and "calcifying globules" (Bonucci, 1971). Sometimes within the cartilaginous and bone matrix they appeared as empty vesicles, other times they appeared to be filled with crystals. These vesicles have been named matrix vesicles. There are currently three major theories as to the origin of matrix vesicles. Of the theories explaining the origin of these vesicles, the most accepted is that which proposes their formation as the result of budding of the plasma membrane (Anderson, 1978; Borg, *et al.*, 1978; Cecil and Anderson, 1978; Wuthier, 1985). It is believed that microvilli on the surface of the chondrocyte (or osteoblast or odontoblast) act as initial budding sites for matrix vesicles into the surrounding matrix. Electron microscopy and chondrocyte cell culture studies have shown that there is great

similarity between matrix vesicles found in culture and those *in vivo* (Boskey, 1978; Wuthier, 1985).

Another theory holds that it is the degeneration of the cellular membrane that releases the cell products responsible for the formation of the matrix vesicles. Support for this theory comes from evidence that the number of matrix vesicles observed decreases as one passes from the reserve zone to the zone of calcification. It is thought that as the plasma membrane breaks up, cell fragments become trapped in the surrounding matrix forming the matrix vesicles. Evidence for this theory comes from studies of pathological calcification processes that occur in atherosclerosis and tumor calcification (Tanimura, *et al.*, 1983; Kim, 1976)

A third theory states that matrix vesicles are made intracellularly in the Golgi complex and extruded through the cell membrane. Support for this theory is weak as there is a lack of intraluminal membranes in the Golgi complex when matrix vesicles are being synthesized at the highest rate.

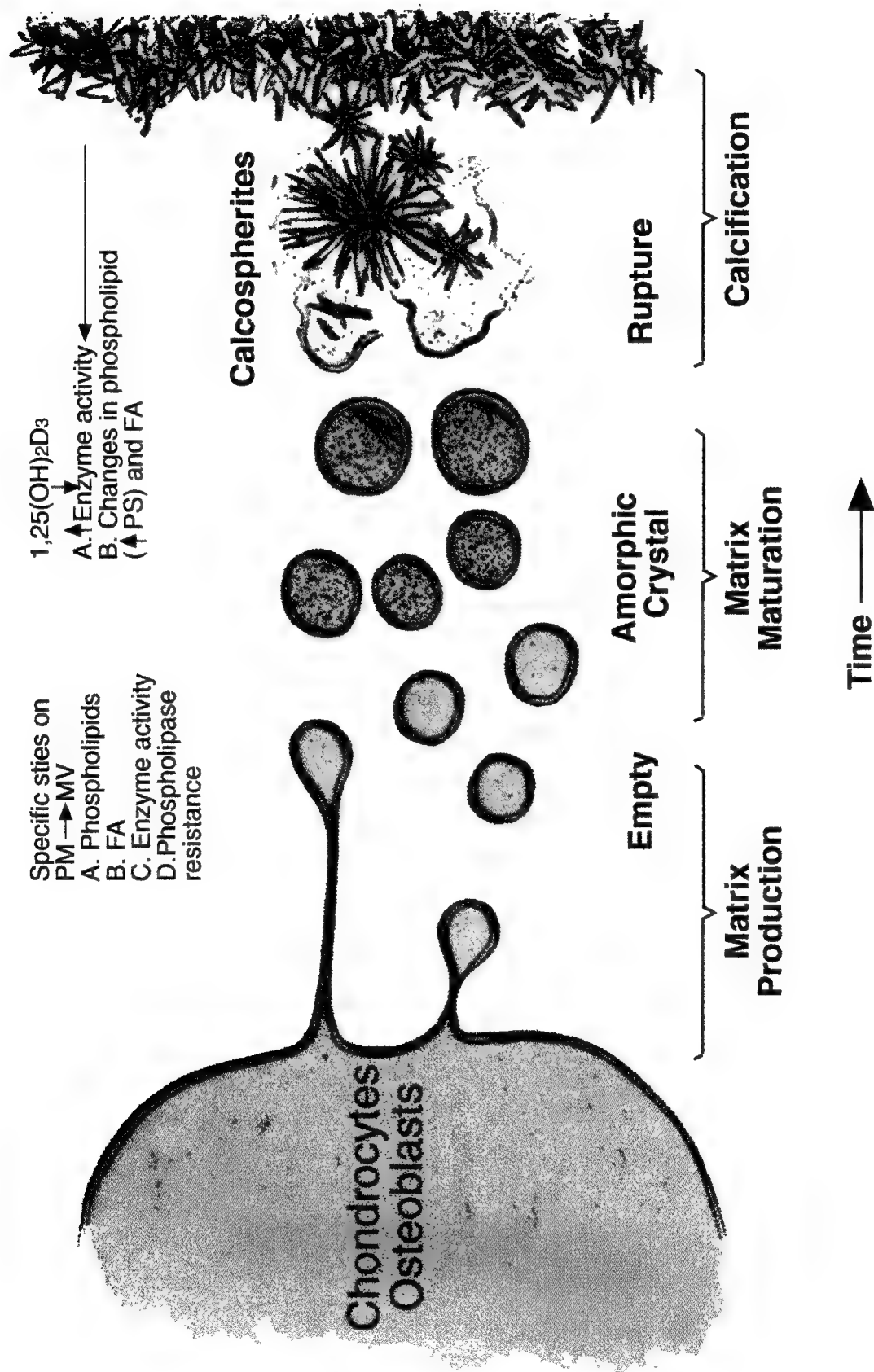
There is some consensus that since the mineralization process is complex, any or all of these processes could occur. It has been proposed that matrix vesicles are a heterogeneous group and that their method of formation is cell specific. During calcification, four categories of matrix vesicles can be discerned: empty, amorphous, crystal, and rupture (Boyan, 1989)(see figure 2). As distance to the calcification front decreases, there is a notable increase in the number of "crystal" filled and "rupture" vesicles and a corresponding decrease in the number of "empty" and "amorphous" types (Bonucci, 1971; Boyan, 1989; Schmitz, *et al.*, 1990).

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**Figure 2:**

Matrix vesicle biogenesis and maturation by chondrocytes or osteoblasts. Illustration by D.B. Boyan.

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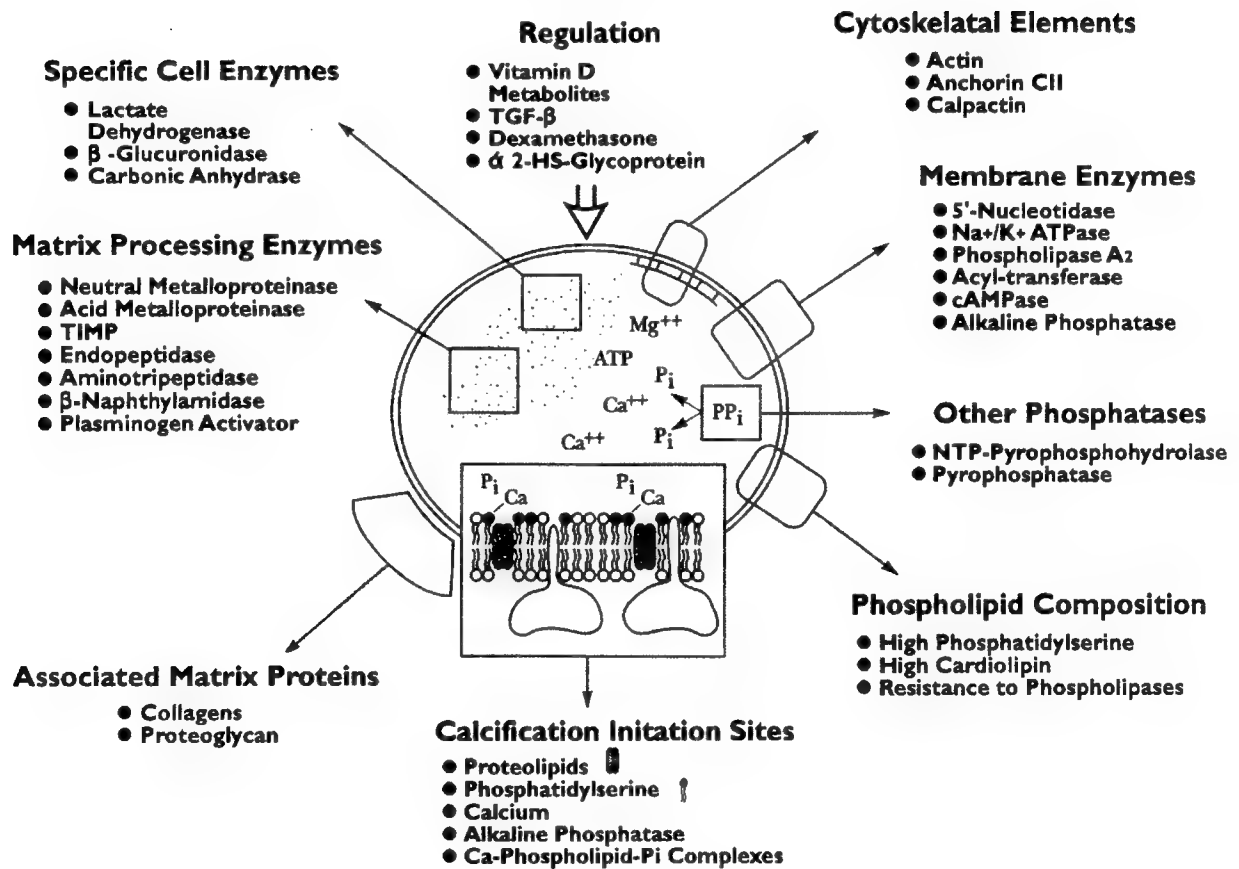
**Figure 3:**

Anatomy of a Matrix Vesicle. Diagram shows structure and biochemistry of a matrix vesicle. Illustration by D.B. Boyan.

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## Anatomy of a Matrix Vesicle



Using computerized morphometry of healing endosteal bone, four stages of matrix vesicle crystal development have been confirmed. In the first stage, the matrix vesicle buds off the cell membrane. The vesicle appears empty with its electron lucent, enzyme rich content. In the next stage, the amorphous stage, vesicles appear to have a uniform electron opaque content due to the increased loading of calcium and phosphate from extracellular fluids. Calcium and phosphate begin to form calcium-phospholipid complexes (CPLX) within the matrix vesicles. It is thought that phosphatidylserine and other phospholipids in the membrane are responsible for the attraction of calcium from extracellular fluids (Wuthier, 1973; Schwartz, *et al.*, 1991)(see figure 3). The third stage, the crystal stage, is characterized by the presence of intravesicular crystal niduses beginning on the internal surface of the vesicle membrane. As mineral deposition continues, hydroxyapatite crystals form within the vesicles either in an equatorial position or within the boundary membrane. Most times a single crystal is found within the vesicle; occasionally multiple crystals exist. The final stage is called the rupture stage. Here the crystals are released to the extracellular environment.

While matrix vesicles are associated with the onset of matrix calcification, it is unclear how the crystals are involved in bulk mineral deposition in the matrix. Calcification appears to occur in areas where focal concentrations of the large hyaluronic acid binding chondroitin sulfate containing proteoglycan and the C-propeptide of type II collagen are increased (Poole, *et al.*, 1989; Boskey, 1981). It appears that the mineralization of the matrix involves proteoglycan degradation, including aggregate breakdown, proteolysis of the core protein, and glycosaminoglycan degradation (Dean, *et al.*, 1992; Poole, *et al.*, 1989; Buckwalter, *et al.*, 1987). Takagi and others (1981), found that through an electron microscopic cytochemical

assay, there is evidence that glycosaminoglycan loss is mediated by matrix vesicles. The main functions of matrix vesicles appears to be the transport of enzymes for matrix modification (Boyan, 1989; Dean, *et al.*, 1992).

Cultured chondrocytes that have entered the hypertrophic stage appear to follow the same intracellular deposition of crystal as do cells found *in vivo*. *In vivo* cartilaginous cells produce proteoglycan rich extracellular matrix containing matrix vesicles. As cells enter the hypertrophic zone there is evidence of crystal formation within the matrix vesicles prior to bulk phase mineralization. In culture, chondrocytes form layers of cells within mounded nodules. Prior to mineralization, the cells become flat on the surface and rounded and hypertrophied at the center. There is evidence that the hypertrophic cells in *in vitro* nodules and those in the growth plate respond to exogenous factors in the same manner.

Matrix vesicles are known to contain phospholipids, alkaline phosphatase, ATPase, and pyrophosphatase (Wuthier, 1975). In addition, matrix vesicles contain active proteases, including neutral and acid metalloproteinase, and plasminogen activator (Dean, *et al.*, 1992). Growth zone chondrocytes show the highest concentrations of these proteoglycan degrading enzymes, indicating that this may be the mechanism by which the more differentiated cell modulates the matrix for calcification (Dean, *et al.*, 1992)."

### C. Alkaline phosphatase

Alkaline phosphatase and phospholipase A<sub>2</sub> specific activities in matrix vesicle membranes are usually high. In particular, alkaline phosphatase has been shown to be more than two fold higher in matrix vesicles than in isolated chondrocyte plasma membranes

(Boyan, *et al.*, 1992a,b,c; Matsuzawa, *et al.*, 1971; Schwartz, *et al.*, 1991; Yang, *et al.*, 1991). This finding is significant because this enzyme is closely associated with the onset of mineralization (Wuthier, 1975; Ali, 1980; Boyan, *et al.*, 1992). However, the exact role of alkaline phosphatase remains unclear (Register, *et al.*, 1984a). One possibility is that it acts to hydrolyze phosphate containing substrates into orthophosphate; orthophosphate is known to be essential for crystal growth within the matrix vesicle (Ali, 1983; Hsu and Anderson, 1978). Alkaline phosphatase is also thought to promote the uptake of calcium and block the inhibitory effects of ATP on hydroxyapatite formation (Ali and Evans, 1981). In any regard, alkaline phosphatase is thought to be a primary marker enzyme for matrix vesicles and to be associated with matrix vesicle-mediated mineral formation.

#### D. Phospholipase A<sub>2</sub>

Phospholipase A<sub>2</sub> levels are found to increase with those of alkaline phosphatase as the calcification front is approached (Wuthier, 1978). Although the precise function of this enzyme in mineralization is unknown, it is thought that it plays a role in the disruption of matrix vesicle membranes during the rupture phase by altering the phospholipid content and fluidity. Boyan and Schwartz (1988) found phospholipase A<sub>2</sub> and alkaline phosphatase activities are regulated by the direct, non-genomic effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (in growth zone chondrocytes) and 24,25-(OH)<sub>2</sub>D<sub>3</sub> (in resting zone chondrocytes). It is thought that this regulation results in changes in phospholipid composition, content, and permeability of the vesicle membrane. Increased levels of Phospholipase A<sub>2</sub> specific activity also used as an indicator for mineralization.

### E. Associated cartilaginous matrix proteins

Numerous other proteins are expressed during endochondral ossification. While some proteins are matrix associated (type II collagen; proteoglycan) others are regulatory factors such as transforming growth factor  $\beta$  (TGF- $\beta$ ), bone morphogenetic protein (BMP), osteocalcin, fibroblast growth factor (FGF), osteoinductive factor (OIF), and platelet derived growth factor (PDGF).

Type II collagen is a major component of the extracellular matrix elaborated by chondrocytes. Type I collagen is produced by fibroblasts, mesenchymal cells, bone producing cells, and fully differentiated calcifying chondrocytes. Thus, it would be expected that there would be changes in type II collagen during the process of endochondral ossification. In the final stages of cellular differentiation and as the distance to the ossification front decreases, an increase in type I collagen occurs.

TGF- $\beta$  cannot spontaneously induce bone formation but it is an essential player in mediating the calcification of the cartilaginous matrix (Centrella, *et al.*, 1992). It appears that TGF- $\beta$  primarily acts to stimulate enzymes associated with calcification and that these effects are maturation dependent (Schwartz, *et al.*, 1993). Synthesis of 1,25-(OH) $_2$ D $_3$  and 24,25-(OH) $_2$ D $_3$  is regulated by TGF- $\beta_1$  in chondrocyte cultures (Schwartz, *et al.*, 1992). TGF- $\beta$  is also produced by these cells and deposited in the extracellular matrix (Schwartz, *et al.*, 1993).

Once expressed, TGF- $\beta$  can further increase DNA proliferative activity and alkaline phosphatase levels perhaps via an autocrine loop (Chen, *et al.*, 1991; Hiraki, *et al.*, 1991). Using platelet and bone derived TGF- $\beta$ , Chen, *et al.* (1991), showed a similar biphasic mitogenic response.

Near the calcification front, Hiraki, *et al.* (1991) found that TGF- $\beta$  reduces matrix accumulation, changes cell morphology, and decreases alkaline phosphate levels in the extracellular matrix. Others have also found that both TGF- $\beta_1$  and TGF- $\beta_2$  increase type I collagen by two- to three-fold and OIF extracellular matrix levels (Chen, *et al.*, 1991; Kubler and Urist, 1991). It has been suggested that while some growth factors like BMP may be responsible for the initial differentiation of mesenchymal cells in osteogenesis, TGF- $\beta$  is responsible for further differentiation of these cells to that of mature, functional osteoblasts (Chen, *et al.*, 1991). It has also been suggested that TGF- $\beta$  may act as a catalyst for the transition of cartilage to bone. In situations where all exogenous matrix factors have been eliminated, TGF- $\beta$  has proven to enhance BMP's ability to stimulate bone formation (Boyan, 1989; Kubler and Urist, 1991).

#### F. Characteristics of BMP

Seven BMPs have been found to date, all of which except BMP-1 are members of the TGF- $\beta$  superfamily (Chen, *et al.*, 1991; Sampath, *et al.*, 1990; Wozney, *et al.*, 1988; Zhou, *et al.*, 1991). The TGF- $\beta$  superfamily also includes inhibin, activin, Mullerian inhibiting substance (MIS), Xenopus Vg-1, and Drosophila decapentaplegic polypeptide (DPP) (Chen, *et al.*, 1991). Of the BMPs, 1,3,4,5, and 7 have been cloned<sup>1</sup>. It is thought that while BMP-2 is primarily responsible for the induction of osteogenesis, other BMP's could augment the

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<sup>1</sup> Alternate or archaic nomenclature for some members of the BMP family:: BMP-2a = BMP-2; BMP-2b = BMP-4; osteogenin = BMP-3; Vgr-1 = BMP-6, Osteogenic protein-1 (OP-1) = BMP-7.

osteogenic capability of BMP-2, either through interaction with the same target cell or by acting on completely different cell populations during the complex process of bone formation (Celeste, *et al.*, 1990). BMP has also been found to be the most potent stimulator of alkaline phosphatase activity described so far (Hiraki, *et al.*, 1991). BMP and OIF are the only two known matrix growth factors that can stimulate cartilage cell differentiation and eventual calcification (Urist, 1989).

The structure of BMP consists of two distinct, but related, subunits that are disulfide-bonded. The disulfide linkage is thought to be an essential part of the biological activity of the growth factor (Urist, *et al.*, 1975). The molecular weight of active human BMP-2 is  $17.0 \pm 0.5$  kDa. It is insoluble in acetone, absolute alcohol, chloroform, methanol, and Triton-X. It is inactivated by heat over 70°C, nitric acid,  $\beta$ -mercaptoethanol, lathyrogens, penicillamine, and ultrasound. It is resistant to collagenase, chondroitinases, amylase A, B, and C, neuraminidase, hyaluronidase, alkaline phosphatase, acid phosphatase, chymopapain, tyrosinase, and thermolysin. It binds to hydroxyapatite and retains its activity after limited proteolysis with pepsin or trypsin (Kubler and Urist, 1991).

#### G. Functions of BMP

BMP action seems to center around chondrocyte differentiation and not cell proliferation (Chen, *et al.*, 1991; Kubler and Urist, 1991; Urist, *et al.*, 1982). Evidence of its ability to promote maturation is demonstrated by the histologic transformation of chondrocytes to cells of a more spherical shape and by their accumulation of proteoglycans both intracellularly and in the cartilage matrix. Cartilaginous volume is noticeably increased under

BMP influence (Hiraki, *et al.*, 1991). In a contrary fashion, TGF- $\beta$  was found to slightly reduce matrix accumulation and changed cell morphology into spindle-like contours in the presence of the fibroblast growth factor (Hiraki, *et al.*, 1991). TGF- $\beta$  also inhibited alkaline phosphatase activity in calcifying chondrocytes, but BMP stimulated it in a dose-dependent manner (Hiraki, *et al.*, 1991). This latter relationship has been noted even more dramatically in mouse MC3T3-E1 osteoblastic cells (Hiraki, *et al.*, 1991). In the presence of optimal doses of TGF- $\beta$ 1 and TGF- $\beta$ 2, BMP will significantly increase alkaline phosphatase activity (Hiraki, *et al.*, 1991). The influence of BMP on connective tissue cells must occur in premitotic or resting cell populations (Sato, *et al.*, 1984).

#### H. Differentiation and Proliferation

*In vivo*, as growth plate fibrocartilage differentiates into calcified cartilage, RNA and soluble protein levels increase as DNA levels decrease. DNA wet weight ratios decrease during cartilage differentiation and calcification. RNA levels, on the other hand, show a significant increase during differentiation (Ketenjian and Arsenis, 1975). Using partially purified BMP in an insoluble non-collagenous protein carrier, Kubler and Urist (1991) found that *in vitro* there was no increase in DNA synthesis in response to even high doses of growth factor. Wuthier, *et al.* (1975) found that in the absence of exogenous growth factors, there was a parallel relationship between chondrocyte incorporation of [ $^3\text{H}$ ]-thymidine (an indicator of DNA synthesis), cellular levels of alkaline phosphatase, and the release of alkaline phosphatase-rich matrix vesicles into the culture medium. "The levels of cellular matrix vesicle alkaline phosphatase were low for the first 12 days of culture, but during the



subsequent 12-day period in which an almost constant rate of [ $^3\text{H}$ ]-thymidine incorporation occurred, cellular alkaline phosphatase activity increased progressively...The peak rate of matrix vesicle (alkaline phosphatase) production occurred just before the time when the rate of [ $^3\text{H}$ ]-thymidine incorporation began to decline" (Wuthier, *et al.*, 1985).

### I. Clinical applications of BMP

*In vivo* experiments have shown that an exposure to 2mg/mL of partially purified BMP is required to observe a significant change in cell morphology. The resulting quantity of bone formation was proportional to the amount of BMP implanted (Boyan, *et al.*, 1992a). Hulth, *et al.* (1988) theorized that BMP diffuses from the ends of broken bones toward the responding cells in the periosteum and marrow. The greatest concentration of bone proteins are found at the ends of bone, leaking both from the fracture line and from the periosteal bone trabeculae that have been stripped of its fibrous covering layers. From these sites, cartilage formation spreads externally and across the fracture gap. The fracture healing is thought to be controlled by the diffusion of BMP from the exposed bone ends. Hulth postulated that BMP is the main initiator of the morphogenetic response for production of bridging callus.

rhBMP-2 has been shown to produce bone *in vivo* that is histologically identical to the type of bone produced from the stimulation of partially purified bovine BMP. However, the time required to produce the bone varied (Wang, *et al.*, 1990; Yamaguchi, *et al.*, 1991). In its pure state, rhBMP-2 appears to contain about a tenth the activity found in partially purified bovine BMP preparations (Celeste, *et al.*, 1990). The actual structure of rhBMP-2 appears to be similar (as with the naturally occurring BMP-2) to TGF- $\beta$ 2, TGF- $\beta$ 1, and rhBMP-4 (Chen,

*et. al*, 1991).

### J. Long-bone and trephine non-union defects

From an orthopaedic perspective, long bone non-union fractures and trephine defects have a poor record for healing once they reach certain proportions. Even though transplantation of autogeneic bone is the best chance for success, the failure rate of autografting non-union fractures and other bone defects is about 30% (Nilsson, *et al.*, 1986). BMP has been used clinically in *in vivo* animal and human studies to regenerate bone. When BMP was used to stimulate new bone, there was a significant increase in the volume of new bone formed over that found with grafting. In Nilsson's study (Nilsson, *et al.*, 1986), it was found that out of 19 segmental defects in dogs, there were no instances of non-union and all defects were repaired in three months. In most cases he found the defect completely bridged in one to two months. Urist (1989) reported that bone regeneration will occur without transplantation of bone marrow if the delivery system consists of other bone matrix non-collagenous proteins and the bone ends are open with free access to hematopoietic bone marrow stroma. Urist surmised that the problem with bone grafting procedures was that cortical bone chips remained in the healing site. When non-collagenous proteins (NCP) were used as a carrier, BMP/NCP was completely resorbed and complete repair occurred in as little as 8 weeks following implantation.

In rabbits, it has been found that implantation of BMP into femoral bone increases metaphyseal bone area by at least 30% (Nilsson, *et al.*, 1985). It has been found that cells from the periosteum differentiate in response to BMP, while osteoprogenitor cells and

osteoblasts respond much less. The authors suggest that the bone marrow cells are the preferred target for BMP and that BMP may play a role in regenerating atrophic alveolar processes.

Human long bone non-union studies also show potential clinical use. In one study of six patients with tibial defects of 3-17 cm, partially purified BMP was placed along the outside of the long bone, as an onlay across the bone defects. Regeneration occurred in a single-stage operation in five patients, the sixth patient required a second operation. In another study, patients, who had experienced an average of three previously unsuccessful bone grafts for femoral non-union defects, were treated with composite bone grafts of cancellous bone marrow and partially purified BMP. All twelve patients healed with an average time of 4.7 months to achieve union (Urist, 1989).

Toriumi, *et al.* (1991) used rhBMP-2 to regenerate mandibles of dogs. The dog mandibles were altered, creating 3cm non-union defects, and implants of rhBMP-2 and inactive dog bone matrix placed into the defects. Dogs in the experimental group were allowed to chew a solid diet after ten weeks. The control group, which employed only the inactive bone matrix, showed minimal or no bone growth. Histologically, 68% of the implanted matrix carrier was replaced with mineralized bone at six months. At six months, the interface between new and old bone was also difficult to ascertain. However, it was found that the new bone was only 60% of the original mandible size. The author suggested that the problem was the carrier used to bring BMP into the area. The author stated that autologous bone grafts have several disadvantages in that: 1) the grafts must have a donor site; 2) there is usually insufficient bone to produce the desired result; 3) surgical sites are prone to infections and resorption; 4) the

donor bone that is needed is rarely rectangular, while the donor obtained usually is (it is like fitting a square peg in a round hole). The difficulty occurs in adapting the bone graft to the complex shape needed for a successful prosthetic placement. It has been suggested that with some of the new synthetic and natural carriers, this concern may soon disappear. The author points out that BMP has several advantages for repairing bony defects. 1) Mandibular continuity can be established without morbidity. 2) Reconstruction of complex regions (anterior alveolar processes, angle and ramus of mandible) is possible.

Resistant trephine defects have been successfully treated with BMP as well. Schmitz, *et al.* (1990) suggested that non-unions develop due to the failure of cells to calcify their matrix. They postulated that this was due to the lack of proper bone-derived growth and differentiation factors levels in the matrix. They found that BMP healed rat calvaria trephine defects from the dural side of the lesion; hydroxyapatite containing matrix vesicles were found to have migrated into the collagen prior to ossification. Ferguson, *et al.* (1987) found that the amount of bone regenerated in calvarial defects was proportional to the amount of BMP implanted and inversely related to the animal's age. He also found that BMP is likely species specific. Interesting to all of the animal and human studies is that no allergic responses to BMP have been found to occur. Host acceptance of rhBMP-2 and purified natural BMP need further investigation.

#### K. Dental Implications

Very little research has been done with respect to ridge augmentation, condylar regeneration, and periodontal defects. Nakashima (1990) has reported on the successful

generation of tubular dentin/osteodentin formation in amputated pulp cavities by partially purified BMP. The author surmised that the osteodentine formed might be substituted for epithelial tissues as a requisite for odontoblastic differentiation by providing a microenvironment and a scaffold for odontoprogenitor cells. Further research needs to be accomplished in dental related use of BMP.

#### L. Bone and Cartilage Models

Much research has been accomplished using chondrocyte models. In a given zone within the growth plate, chondrocytes are only of one cell type. This makes the isolation of distinct phenotypes possible (Boyan, *et al.*, 1989). Bone, in contrast, is relatively heterogenous. Not only are the different stages of cell differentiation intermixed, but the tissue is mineralized and highly vascular making the isolation of matrix vesicles very difficult. Because chondrocyte phenotypes are both spatially and temporally separated, these cells are ideal for the study of endochondral ossification.

## **II. METHODS AND MATERIALS**

### **A. Chondrocyte Culture**

Using a previously described method (Boyan, *et al.*, 1992b), chondrocytes were isolated from cells obtained from resting and growth zone cartilage of the costochondral junction of 125 g male Sprague-Dawley rats. Rib cages were removed by sharp dissection and placed in serum-free Dulbecco's Modified Eagle's Medium (DMEM). Ribs were then separated, defleshed, and the resting zone and adjacent growth zone cartilages removed. Cartilages were separated according to their zone of origin. Care was taken to dissect out intervening tissue to limit cross contamination of cell types. The DMEM was removed with two twenty minute washes of Hank's Balanced Salt Solution (HBSS). This was followed by sequential incubations in 1% trypsin (Gibco, Grand Island, NY) for one hour and 0.02% collagenase (Sigma, St. Louis, MO) for three hours. All enzymes were prepared in HBSS. After enzymatic digestion, the cell and tissue debris were separated with a sterile 40-mesh nylon suction apparatus. The filtrate was centrifuged at 500 X g for 10 minutes to recover the cells which were then resuspended in DMEM and counted. The cells were placed in T-75 flasks at a density of 10,000 cell/cm<sup>2</sup> for resting zone chondrocytes and 25,000 cell/cm<sup>2</sup> for growth zone chondrocytes. The cells were incubated in DMEM containing 10% fetal bovine serum (FBS), 50µg/mL vitamin C and 1% penicillin-streptomycin-fungizone in 5% CO<sub>2</sub> at 37<sup>0</sup> C (100% humidity). The medium was changed every seventy two hours until the cells reached confluence; cells reached confluence within an average of seven days. Cells were subcultured for a maximum of three passages to ensure retention of phenotype.

Human recombinant bone morphogenetic protein (rhBMP-2; obtained from Genetics

Institute, Cambridge, MA) was tested at physiological and pharmacological doses ranging from 0.05 to 100 ng/mL. Each experiment included controls which were not stimulated with rhBMP-2. All experimental cells were incubated to confluence a fourth time as described below.

#### B. Determination of Alkaline Phosphatase Specific Activity

At confluence, third passage cells were seeded into 24-well plates at 10,000 cells/cm<sup>2</sup> for resting zone chondrocytes and 25,000 cells/cm<sup>2</sup> for growth zone chondrocytes and incubated until confluence was obtained. Cells were treated with rhBMP-2 (0.01 to 100 ng/mL) for 24 and 48 hours. At the end of the incubation, the medium was removed and the cells washed with phosphate buffered saline (PBS). The cells were then broken loose with a scraper and the cells remaining in the well collected with another wash of PBS. Both samples were pooled and the plasma membranes (for each cellular phenotype) and matrix vesicles were isolated by centrifuging each sample at 1,000 X g for 30 minutes. The resulting supernatant was discarded and the pellet washed with 500 $\mu$ L PBS. The membranes for each cell layer were collected after a second centrifugation and the pellet resuspended in 0.05% Triton X-100 (Hale, *et al.*, 1986). Each sample was frozen and thawed five times to disrupt the membranes remaining in the pellet. Alkaline phosphatase and protein levels were measured in the resulting matrix vesicles and cell plasma membranes.

**Alkaline Phosphatase.** Alkaline phosphatase (orthophosphoric monoester phosphohydrolase alkaline [E.C. 3.1.3.1.]) was measured as a function of the release of *para*-nitrophenol from *para*-nitrophenyl phosphate at pH 10.2 (Bretaudiere, *et al.*, 1984). 50 $\mu$ L of

each sample was assayed in duplicate using 96 well culture plates by mixing with 50 $\mu$ L 2-amino-2-methyl-1-propanol (AMP) buffer, covering the plates with parafilm and incubating at 37°C until the color change occurs (up to 3 hours). Following the color change, 100 $\mu$ L of 1M NaOH was added to each well to stop the reaction. Once the plate had reached room temperature, it was read on an ELISA plate reader (BioRad, Inc., Richmond, CA) at 405 nm. Data were reported as enzyme units/mg protein (specific activity) for individual experiments or as treatment/control ratios for comparison among experiments.

**Total Protein.** Protein was measured with the Micro BCA Protein Assay Reagent (Pierce Chemical Co., Rockford IL) using bovine serum albumin as the standard. This test is based on the biuret reaction where  $\text{Cu}^{+2}$  is reduced in an alkaline medium to produce  $\text{Cu}^{+1}$ , which then reacts with two molecules of bicinchoninic acid to form a purple, water soluble product with a strong absorbance at 562 nm (Smith *et al.*, 1987). Duplicate 100  $\mu$ L sample were placed in 96 well plates and 100 $\mu$ L reagent added. The plates were incubated for one hour at 60°C prior to reading on an ELISA plate reader. Results were reported as mg protein/sample.

### C. Determination of [ $^3\text{H}$ ]-Thymidine Incorporation

[ $^3\text{H}$ ]-Thymidine incorporation was used to determine DNA synthesis by resting zone and growth zone chondrocytes in response to treatment with rhBMP-2. The assay for DNA synthesis was identical to that for measuring RNA with the following two exceptions: samples were incubated in experimental media for 20 hours instead of 5, and 50  $\mu$ L [ $^3\text{H}$ ]-thymidine (from a 5 $\mu$ Ci/mL stock) was added to the samples for 4 hours. A second group of confluent



cells was placed in quiescence (resting zone and growth zone cells were preincubated for 48 hours in DMEM containing 1% FBS. Cell proliferation rates were determined by measuring DPM per well.

#### D. Determination of Collagenase Digestible and Non-Collagenase Digestible Protein Production

To determine the effect of rhBMP-2 on resting zone and growth zone cell production of collagen, incorporation of labeled proline into collagenase digestible proteins (CDP) and non-collagenase digestible protein (NCP) production was measured (Beresford, *et al.*, 1986; Raisz, *et al.*, 1979).

In procedures described earlier (Schwartz, *et al.*, 1989), third passage resting zone and growth zone chondrocytes were cultured in 6 well plates. Growth zone chondrocyte cells were plated at densities of 10,000 cells/cm<sup>2</sup> and resting zone cells at 25,000 cells/cm<sup>2</sup> and then incubated for 24 hours in experimental media (with rhBMP concentrations ranging from 0.01 to 100 ng/mL). The cells were labeled for 24 hours with 1mL [<sup>3</sup>H]-proline (5μCi/mL) in full media.

The determination of collagenase digestible and non-collagenase digestible protein production was performed as previously described (Raisz, *et al.*, 1978; Raisz, *et al.*, 1979; Huey, *et al.*, 1980 ). For each sample, the medium was removed and saved for protein extraction later. The cell layer was harvested using two 200μL portions of 0.2 N NaOH. Both the medium and cell layer derived proteins were independently precipitated using 0.1mL of 100% trichloroacetic acid (TCA) containing 10% tannic acid (Peterkofsky, *et al.*, 1971). Each

sample was then centrifuged at 400 X g for 10 minutes to isolate the precipitate. 10% TCA/1% tannic acid was added to each pellet and the pellets from the medium and cell layer proteins were pooled. Samples were then washed three times with 500 $\mu$ L aliquots of 10% TCA/1% tannic acid. Pellets were then washed twice with 100  $\mu$ L aliquots of ice cold acetone (Helenius, 1971) and the final pellet was dissolved in 500 $\mu$ L 0.05M NaOH. Protein content of each pellet was determined by the Micro BCA Protein Assay Reagent as described above. Total protein synthesis, as measured by proline incorporation was determined by placing 50 $\mu$ L of each sample into scintillation vials containing 10 mL of scintillation fluid (Ready Protein +, Beckman Instruments Inc., Fullerton, CA) and liquid scintillation counting.

Collagenase digestible and non-collagenase digestible protein production were then determined as follows. 500 $\mu$ L of solution containing 25 units of collagenase (Sigma) in 60  $\mu$ M HEPES buffer (pH 7.2) [N-2 hydroxyethyl piperazine-N-2-ethano-sulfonic acid], 1.25 $\mu$ M of N-ethylmaleimide (NEM), and 0.25 $\mu$ M CaCl<sub>2</sub> was added to 200  $\mu$ L sample as prepared above and incubated for 4 hours at 37°C (the collagenase used had no proteolytic activity on non-collagen substrates). At the end of the incubation, 500 $\mu$ L of 10% TCA/1% tannic acid was added (for 5 minutes) to stop the reaction. Samples were then centrifuged at 400 X g for 5 minutes at 4° C. The supernatant containing the radioactivity associated with collagenase digestible protein (CDP) was transferred to 10 mL scintillation cocktail (Ready Protein +) and set aside. The precipitate was resuspended in 500  $\mu$ L of 5% TCA /0.25% tannic acid for 12 to 20 hours at -20° C. The suspensions were recentrifuged and the supernatant transferred to the vial containing the CDP. To quantitate non-collagenase digestible protein (NCP), the remaining pellets were resuspended in 1mL 5% TCA/0.25 % tannic acid, transferred to 10

mL scintillation cocktail (Ready Protein +), and counted in a liquid scintillation counter. The percent collagen synthesis was calculated by using a factor of 5.4 times the proline in NCP to correct for the relative abundance of proline in collagen and non-collagen protein (Raisz, *et al*, 1978).<sup>2</sup>

#### E. Determination of [<sup>3</sup>H]-Uridine Incorporation

Total RNA synthesis was determined by measuring the incorporation of [<sup>3</sup>H]-uridine. Confluent resting zone and growth zone cell cultures were subcultured into 96 well plates. Cell plating densities were 25,000 cells/cm<sup>2</sup> for growth zone chondrocytes and 10,000 cells/cm<sup>2</sup> for resting zone chondrocytes. The cells were incubated for 48 hours with full medium (150  $\mu$ L per well) to allow for cell recovery and attachment. Once the cells reached confluence, the medium was removed and replaced with 150  $\mu$ L experimental medium containing rhBMP concentrations ranging from 0.01 to 100 ng/mL. (Schwartz, *et al.*, 1989).

After 5 hours of incubation with experimental medium, 50  $\mu$ L [<sup>3</sup>H]-uridine (from a 5 $\mu$ Ci/mL stock) was added to each well and the incubation continued for an additional 2 hours. At the end of the incubation, the cells were washed two times with 150  $\mu$ L PBS. The cells were then fixed by washing three times with 150 $\mu$ L of cold 5% TCA. Following the third wash, cells were cooled to 4°C for 30 minutes. The TCA was removed and the wells allowed to air dry. The fixed cells were then resuspended in 200  $\mu$ L 1% SDS for 30 minutes at room temperature. The samples were added to scintillation vials containing 10 mL of scintillation

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<sup>2</sup> % collagen synthesized = [(CDP dpm/ $\mu$ g dry wt)/5.4]/[(CDP dpm/ $\mu$ g dry wt)/5.4] + NCP dpm/ $\mu$ g dry wt] X 100.

cocktail (Ready Protein +) and counted in a liquid scintillation counter.

#### F. Statistical Analysis

Each data point reported represents the mean  $\pm$  standard error of the mean for at least six cultures in a particular experiment. Observations for each experiment were validated by repeating each experiment a minimum of two times. Statistical significance was determined by comparing each experimental group to the untreated control using Student's t-test as modified by Bonferroni (Bonewald, *et al.*, 1990). Significant differences were determined by analysis of variance. Differences were considered significant at  $p < 0.05$ .

In studies of alkaline phosphatase, where treatment was compared to control, the statistical analysis was handled differently. A mean from each experimental group (n of 6) was first determined. From these means, the standard error was calculated for a given treatment dose. The mean for each dose was then compared to the mean of the untreated control with the Wilcoxon's paired test.

### III. RESULTS

#### A. Alkaline Phosphatase

The cell layers of both resting zone and growth zone cultures showed a significant increase in alkaline phosphatase specific activity after treatment for 24 or 48 hours with doses of rhBMP-2 ranging from 0.05 to 100 ng/mL. The data in figures 4 to 7 are shown as either specific activity ( $\mu\text{mol Pi/mg protein/minute}$ ) or treatment/control ratios of the specific activity.

Growth zone cells after 24 or 48 hours of treatment showed significant 1.2 to 2.0 fold stimulations in specific activity (figures 4 and 5) at rhBMP-2 concentrations of 12.5ng/mL-100ng/mL that was dose dependent.

Resting zone cells showed a biphasic response in alkaline phosphatase specific activity after treatment with rhBMP-2 (figures 6 and 7). An initial significant increase in alkaline phosphatase specific activity was found between the rhBMP-2 concentrations of 0.2-0.8ng/mL at both 24 and 48 hours, with peaks at 0.2ng/ml and 0.4ng/ml for the 48 and 24 hour groups respectively. A second significant increase was found between rhBMP-2 concentrations of 25-100ng/ml for the 48 hour group and between 50-100ng/ml for the 24 hour group (figure 6). In both time groups, the initial increase (0.2-0.8ng/mL) exhibited a two fold elevation over control (figure 7). The fold increase in alkaline phosphatase specific activity was about equal at both concentration ranges where statistical significance was observed (figure 7).

#### B. [ $^3\text{H}$ ]-Thymidine Incorporation

At quiescence, both resting zone and growth zone cultures showed significant dose

dependent increases in [ $^3\text{H}$ ]-thymidine incorporation over the control groups after treatment with 12.5 - 100ng/mL rhBMP-2 (figure 8). In the dose range that produced significant increases in [ $^3\text{H}$ ]-thymidine incorporation, growth zone chondrocytes showed a greater fold increase than the resting zone chondrocytes.

For the 24 and 48 hour time courses, the cells were not placed into quiescence prior to treatment with rhBMP-2. Growth zone [ $^3\text{H}$ ]-thymidine incorporation showed no significant change over the control groups at either the 24 or 48 hour time points (figure 9). Resting zone cells, however, did show a significant dose dependent increase in [ $^3\text{H}$ ]-thymidine incorporation with rhBMP-2 concentrations above 12.5ng/mL (figure 10). There was no difference between the 24 and 48 hour time intervals observed for the resting zone cells.

#### C. Collagenase Digestible and Non-collagenase Digestible Protein Stimulation

In growth zone and resting zone chondrocyte populations, there was a significant increase over control in CDP and NCP production at concentrations of 3.1 - 25ng/mL rhBMP-2 (figure 11). Levels above and below this range showed no significant difference from the control. Growth zone cells showed a two fold greater NCP production than the resting zone cells at all rhBMP-2 concentrations (figure 12). Just the opposite appeared to be true with the collagenase digestible proteins. Here the resting zone cell population showed a significantly greater protein production than the growth zone cell groups.

#### D. [ $^3\text{H}$ ]-Uridine Incorporation

In resting zone cells there was a significant increase in [ $^3\text{H}$ ]-uridine incorporation at

rhBMP-2 concentrations of 50-100ng/mL (figure 13). There was no significant difference in the amount of [ $^3$ H]-uridine incorporation based on whether the resting zone chondrocytes were treated for 24 and 48 hours. With growth zone cells, there was also a significant increase over control, but starting at lower concentrations of BMP-2 (25ng/mL) and only in the 24 hour group; a significant increase over control was found in the 25-100ng/mL rh BMP-2 range (figure 14). Also notable was the significantly higher amount of [ $^3$ H]-uridine incorporated in the 24 hour group over that found in the 48 hour group; about a 4 fold difference.

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**Figure 4:**

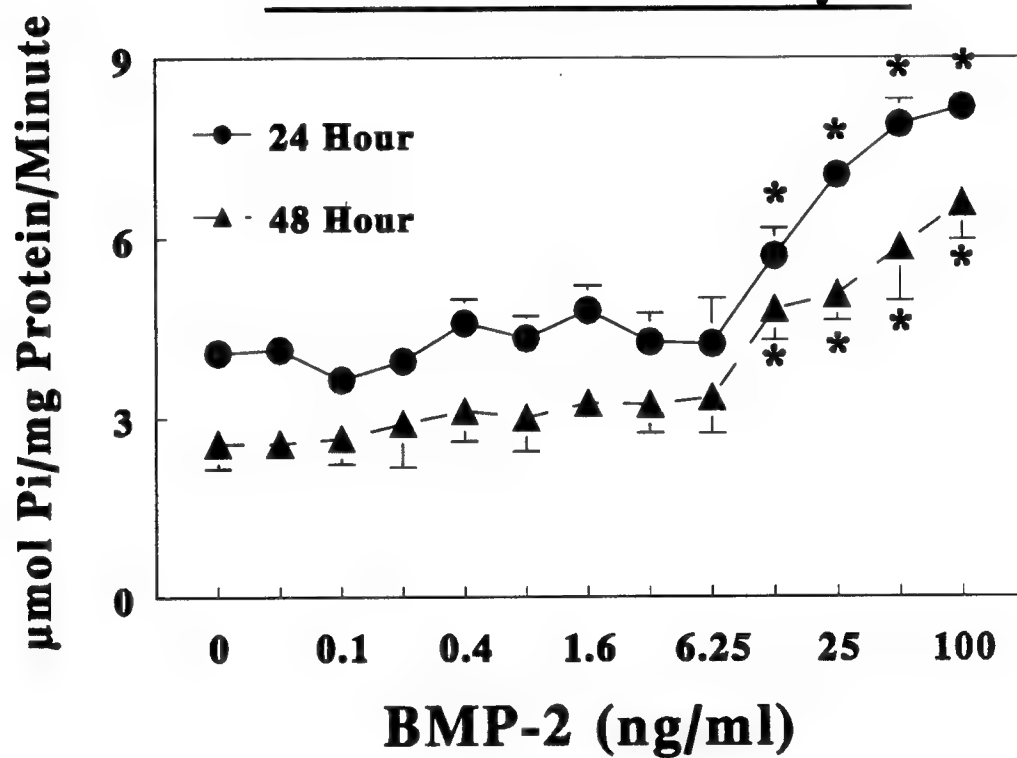
The effect of varying amounts of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on alkaline phosphatase specific activity in cell layers isolated from growth zone chondrocyte cultures, after 24 or 48 hours of treatment.

Values are the mean  $\pm$  SEM for an n of 6. \*p < 0.05 for sample versus control. Each experiment was repeated 2 times.

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## ALPase Specific Activity Growth Zone Chondrocytes



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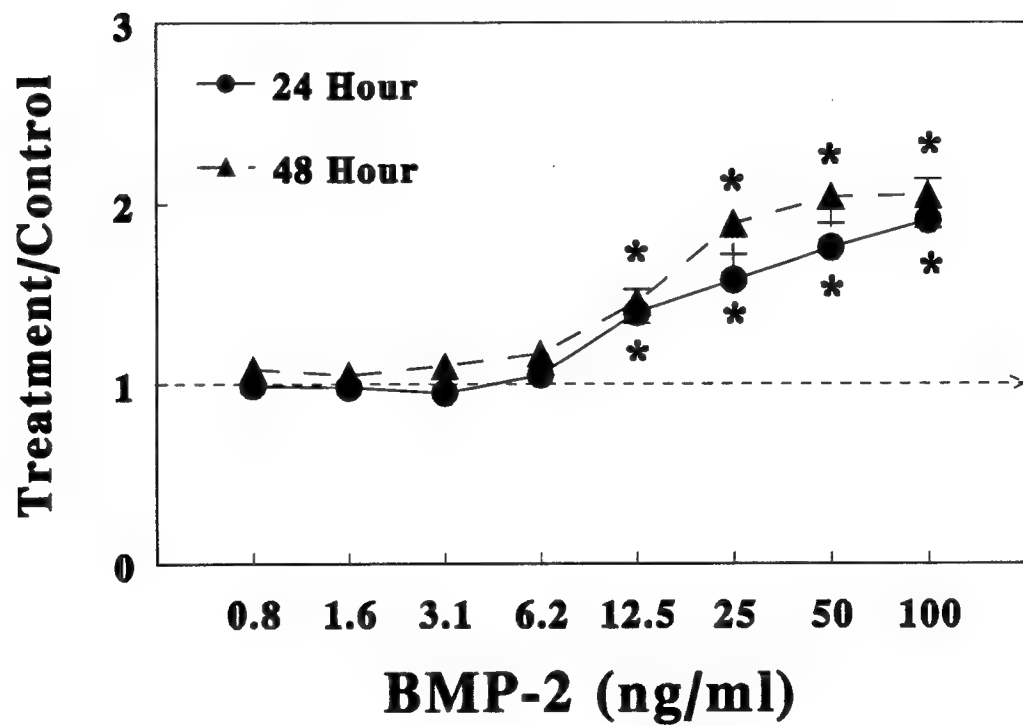
**Figure 5:**

The effect of varying amounts of rhBMP-2 on alkaline phosphatase specific activity in cell layers isolated from growth zone chondrocyte cultures after 24 or 48 hours of treatment. The data are expressed as treatment/control ratios for 6 experiments.

Values are the mean  $\pm$  SEM for each rhBMP-2 concentration group. \*p < 0.05 for each treated sample versus control. ALPase: alkaline phosphatase.

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## ALPase Specific Activity Growth Zone Chondrocytes



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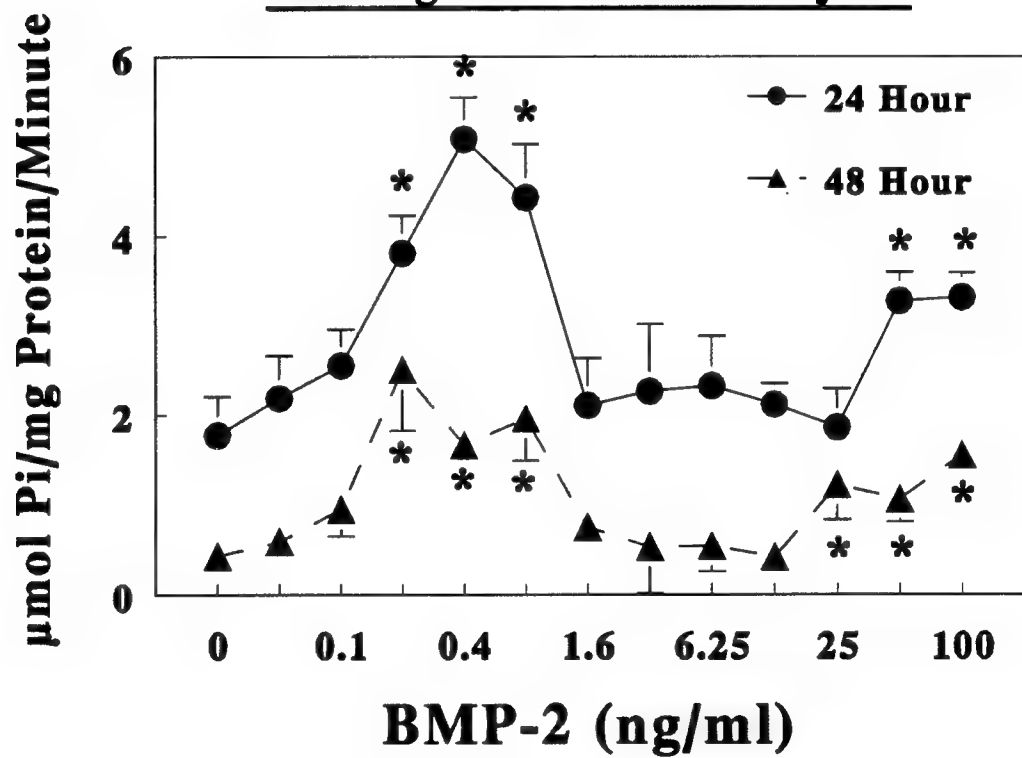
**Figure 6:**

The effect of varying amounts of rhBMP-2 on alkaline phosphatase specific activity in cell layers isolated from resting zone chondrocyte cultures after 24 or 48 hours of treatment.

Values are the mean  $\pm$  SEM for each rhBMP-2 concentration group. \*p < 0.05 for sample versus control. Each experiment was repeated 2 times. ALPase: alkaline phosphatase.

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## ALPase Specific Activity Resting Zone Chondrocytes



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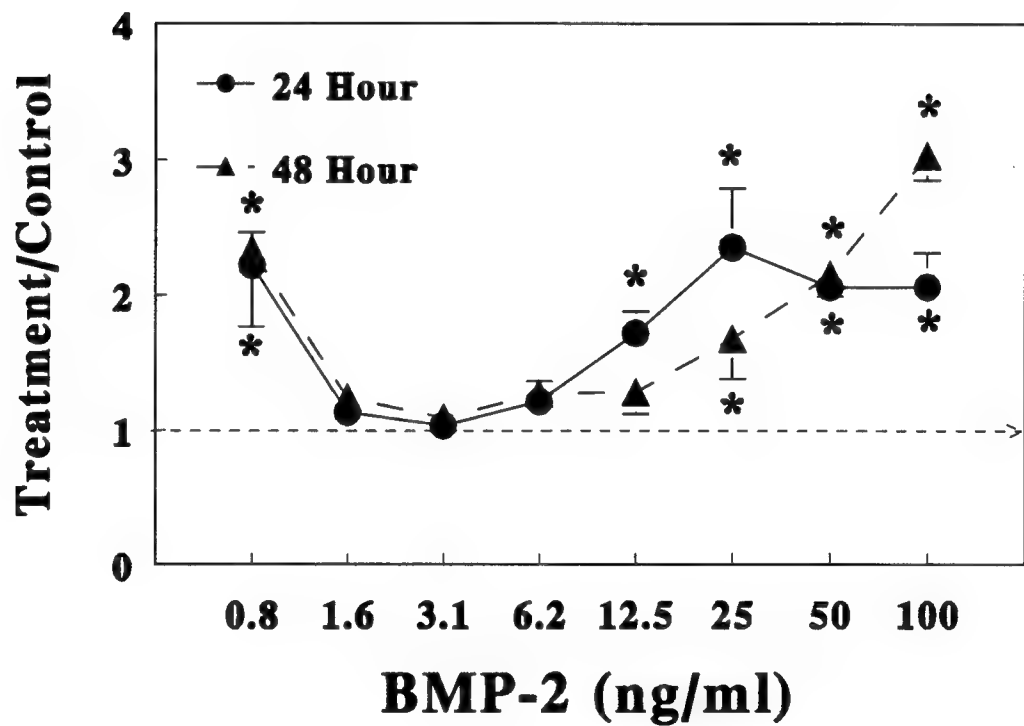
**Figure 7:**

The effect of varying amounts of rhBMP-2 on alkaline phosphatase specific activity in cell layers isolated from resting zone chondrocyte cultures after 24 or 48 hours of treatment. The data are expressed as treatment/control ratios for 6 experiments.

Values are the mean  $\pm$  SEM for an n of 6. \*p < 0.05 for each treated sample versus control. ALPase: alkaline phosphatase.

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## ALPase Specific Activity Resting Zone Chondrocytes



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**Figure 8:**

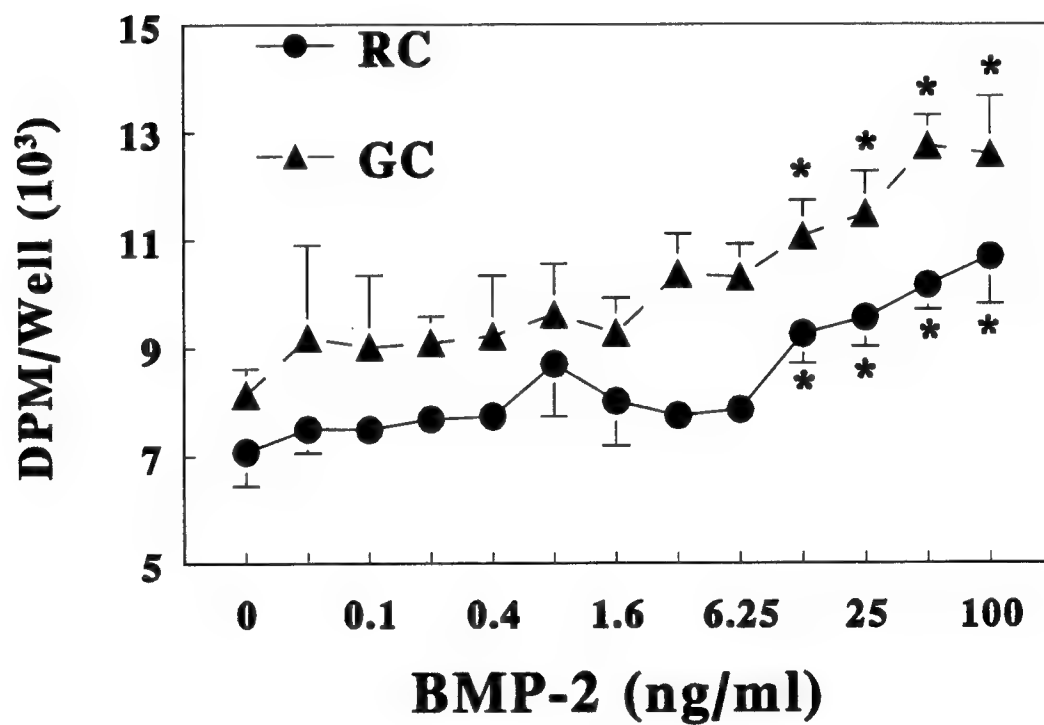
The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated thymidine incorporation by quiescent growth zone (GC) and resting zone (RC) chondrocytes.

Values are the mean  $\pm$  SEM for an n of 6. \*p < 0.05 for each treated sample versus control. Each experiment was repeated 2 times.

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## [<sup>3</sup>H]-Thymidine Incorporation



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**Figure 9:**

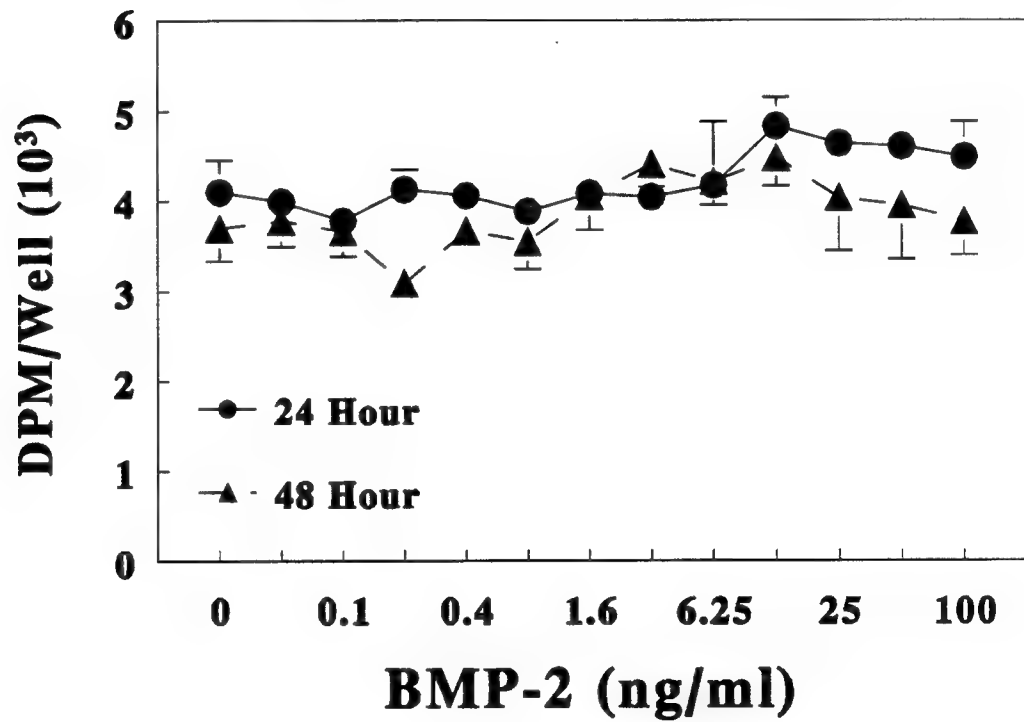
The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated thymidine incorporation after 24 or 48 hours in confluent, non-quiescent fourth passage costochondral growth zone chondrocytes.

Values are the mean  $\pm$  SEM for an n of 6. \*p < 0.05 for each treated sample versus control. Each experiment was repeated 2 times.

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# **[<sup>3</sup>H]-Thymidine Incorporation**

## **Growth Zone Chondrocytes**



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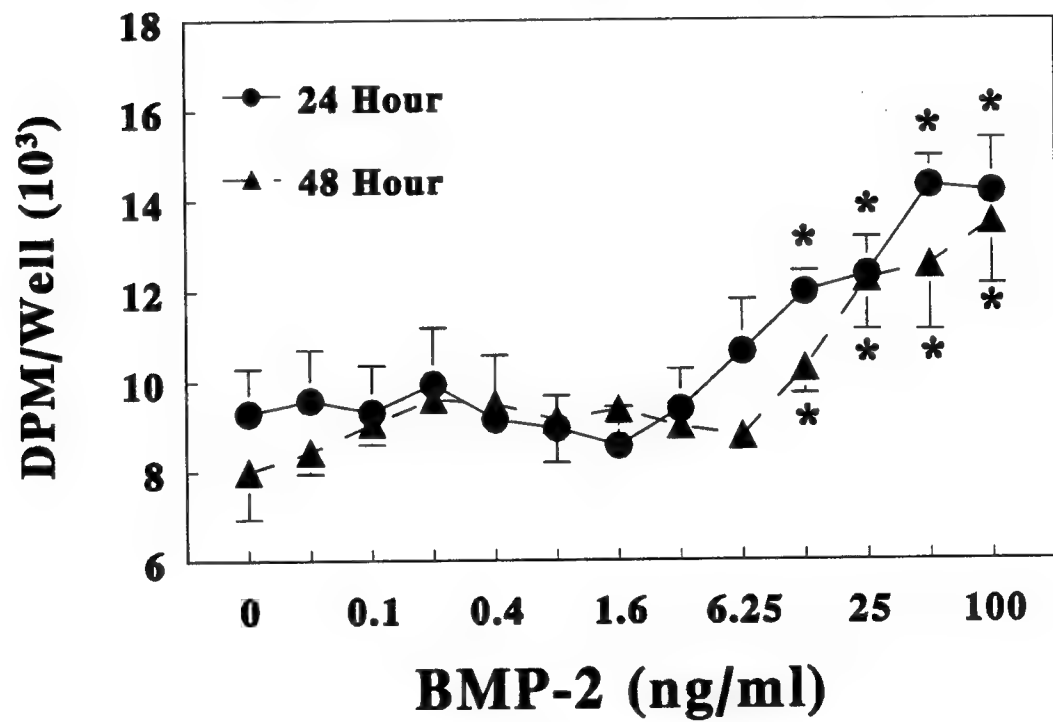
**Figure 10:**

The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated thymidine incorporation after 24 or 48 hours in confluent, non-quiescent fourth passage costochondral resting zone chondrocytes.

Values are the mean  $\pm$  SEM for an n of 6. \*p < 0.05 for each treated sample versus control. Each experiment was repeated 2 times.

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# **[<sup>3</sup>H]-Thymidine Incorporation Resting Zone Chondrocytes**



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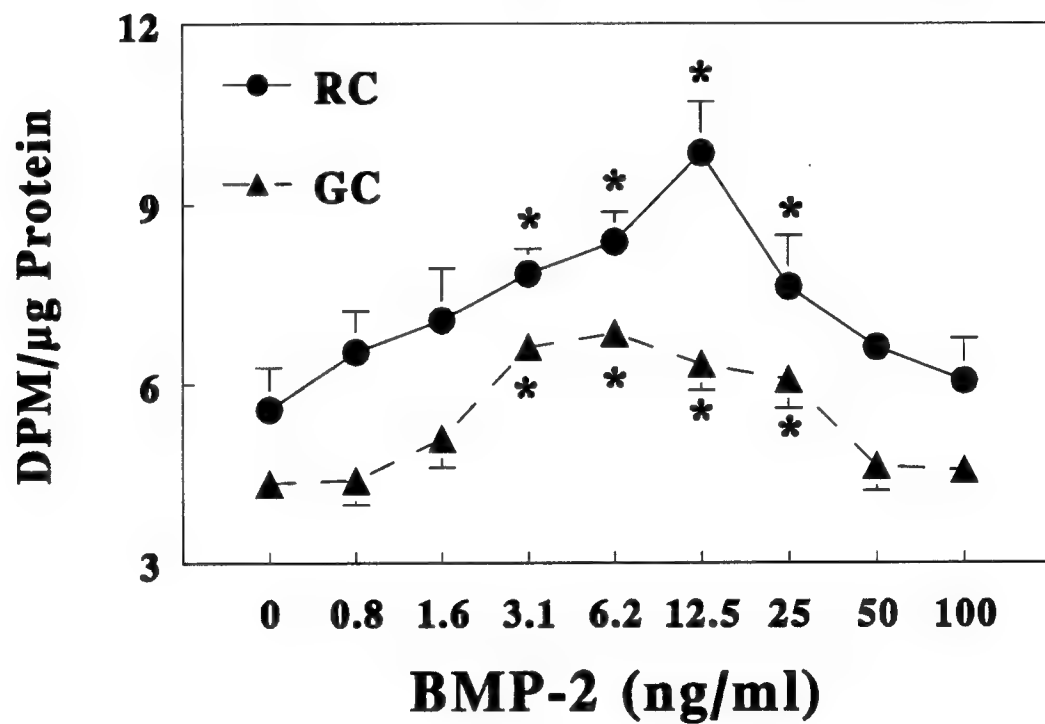
**Figure 11:**

The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on tritiated proline incorporation into collagenase digestible protein. Confluent, fourth passage costochondral growth zone (GC) and resting zone (RC) chondrocytes were examined.

Values are the mean  $\pm$  SEM for an n of 6. \* $p < 0.05$  for each treated sample versus control. Each experiment was repeated 2 times.

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### **[<sup>3</sup>H]-Proline Incorporation Collagenase Digestible Protein**



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**Figure 12:**

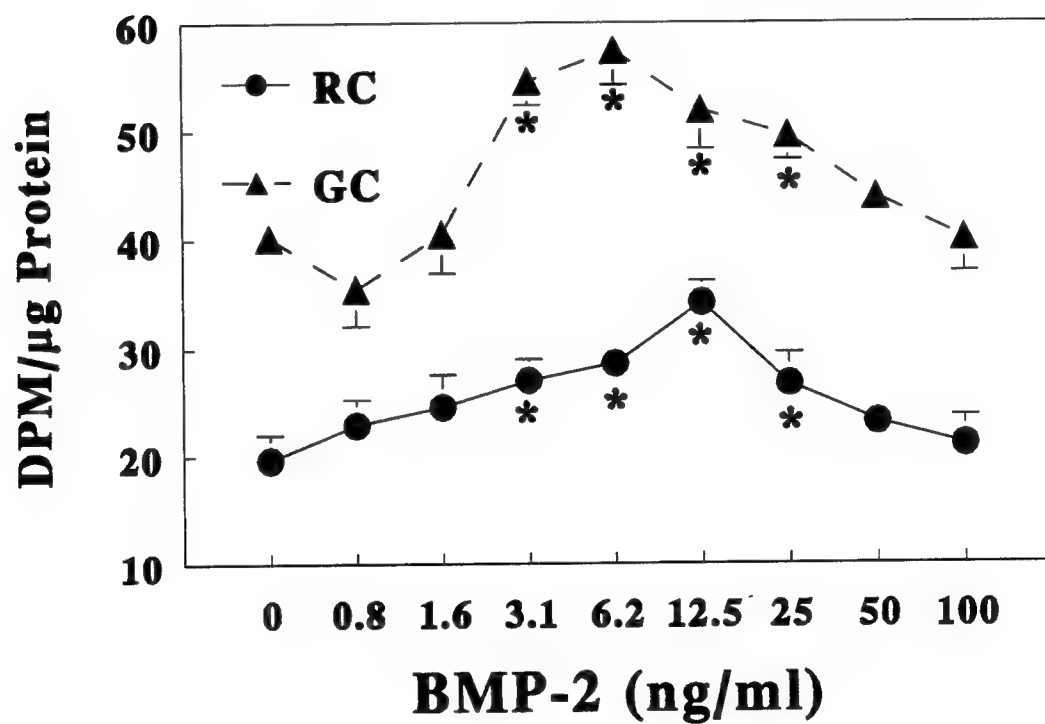
The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated proline incorporation into non-collagenase digestible protein. Confluent, fourth passage costochondral growth zone (GC) and resting zone (RC) chondrocytes were examined.

Values are the mean  $\pm$  SEM for an n of 6. \*p < 0.05 for each treated sample versus control. Each experiment was repeated 2 times.

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### **[<sup>3</sup>H]-Proline Incorporation** **Non-collagenase Digestible Protein**



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**Figure 13:**

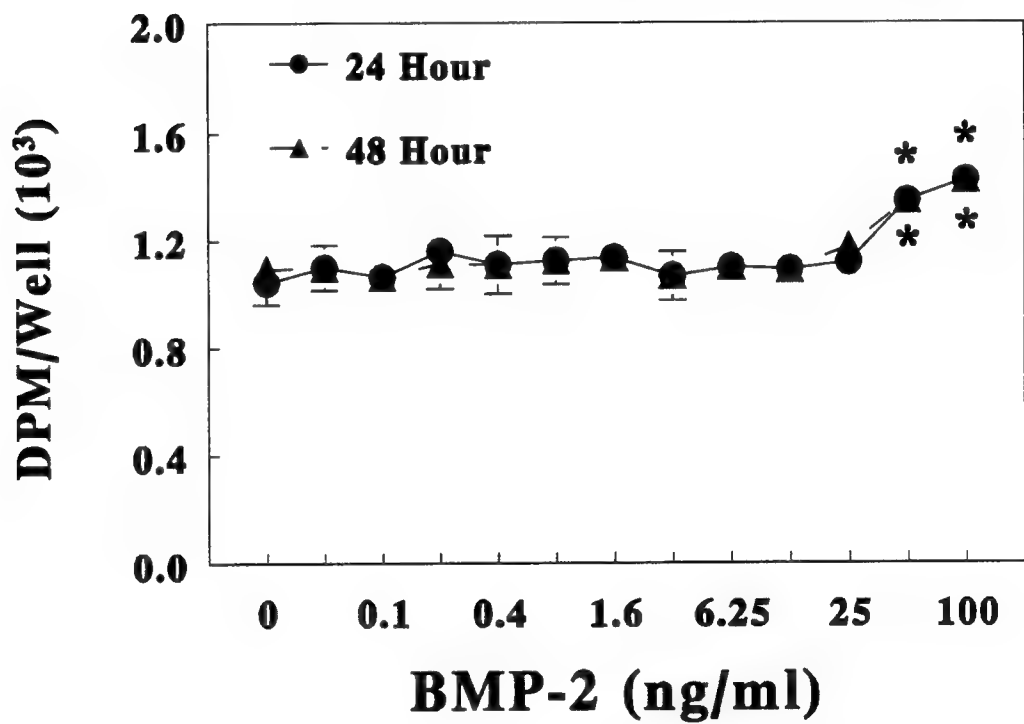
The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated uridine incorporation by confluent, fourth passage costochondral resting zone chondrocytes.

Values are the mean  $\pm$  SEM for an n of 6. \*p < 0.05 for each treated sample versus control. Each experiment was repeated 2 times.

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# **[<sup>3</sup>H]-Uridine Incorporation**

## **Resting Zone Chondrocytes**



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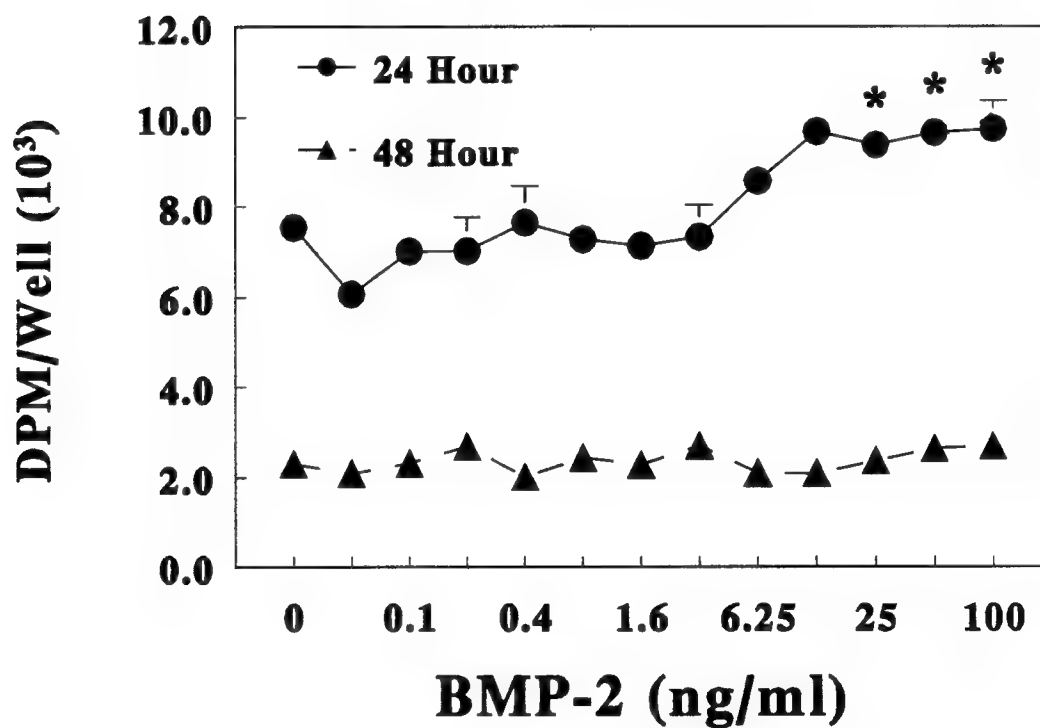
**Figure 14:**

The effect of recombinant human bone morphogenetic protein - 2 (rhBMP-2) on tritiated uridine incorporation by confluent, fourth passage costochondral growth zone chondrocytes.

Values are the mean  $\pm$  SEM for an n of 6. \* $p < 0.05$  for each treated sample versus control. Each experiment was repeated 2 times.

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### **[<sup>3</sup>H]-Uridine Incorporation Growth Zone Chondrocytes**



#### IV. DISCUSSION

Of all the known forms of BMP, BMP-2 is clearly the human analog of the protein present in bovine bone that Urist first discovered (Wozney, *et al.*, 1988). It is one of only a few agents found thus far that can stimulate mesenchymal cells to form cartilage and bone independent of other growth factors. A main problem with most of the early work on BMP was that crudely or partially purified preparations were used. We know that in the bone and cartilage matrix there are a plethora of proteins, many of which can influence ossification at specific stages of cell differentiation. For instance, we know that vitamin D<sub>3</sub>, platelet growth factor-2, cartilage induction factor-A, and transforming growth factor- $\beta$  can variegate BMP's effect on chondrocytes calcifying their matrix (Boyan, *et al.*, 1992b; Wozney, *et al.*, 1988). The question becomes, what is the mechanism behind BMP induced bone formation and how does it interact with the other matrix proteins present?

With the cloning of human BMP-2 (BMP-2) we are closer to an answer. We know from the literature that BMP-2's molecular structure and ability to induce calcification in soft tissue is very similar to that found in the crudely extracted forms of BMP. With a BMP molecule free of exogenous matrix factors, specific BMP functions should be, and have been, easier to discern.

It has been speculated that BMP may be a primary agent promoting the differentiation of mesenchymal cells into chondrocytes. In the cascade of endochondral ossification, these newly differentiated immature resting zone chondrocytes would, under the influence of BMP, continue to proliferate and then differentiate into growth zone chondrocytes. Chen, *et al.*, have

hypothesized that transforming growth factor- $\beta$  may then be the primary matrix factor responsible for the differentiation of growth zone chondrocytes into osteoblasts (Chen, *et al.*, 1991). However, it is equally probable that transforming growth factor- $\beta$  promotes early chondrogenic differentiation and BMP stimulates terminal calcified cartilage differentiation. The calcified matrix then provides a scaffold for osteogenesis via a new crop of mesenchymal cells.

In osteoblast like (ROB) cells, Chen, *et al.*, (1991) found that even though others have reported an increase in both cell number and alkaline phosphatase levels with rhBMP-2, they were able to stimulate a response only at very high doses. To eliminate variability among cell populations due to differences in seeding densities or degree of confluence at time of exposure to BMP, we compared treatment control ratios for alkaline phosphatase levels from 5 experiments. The results indicate that the degree of response to BMP-2 for alkaline phosphatase specific activity appeared to be maturation dependent. In growth zone chondrocyte cultures an increase in alkaline phosphatase was found only at the higher doses. In resting zone chondrocyte cultures, however, a marked response was seen at both higher (as in the growth zone cultures) and at lower concentrations of rhBMP-2 suggesting that resting zone chondrocytes are the more sensitive to BMP-2. It also appears that the longer the cultured chondrocytes were exposed to BMP (24 vs 48 hours), the lower the alkaline phosphatase specific activity. It could be postulated that BMP affects chondrocytes the most at earlier stages in the ossification cascade and that as the chondrocytes mature, differentiate, and proliferate they release BMP into the surrounding matrix, resulting in an autocrine effect on the cells. It would make sense then that the more matured chondrocytes would become less

sensitive to BMP. Indeed, recent unpublished data from our lab, found rhBMP-2 positively autoregulates levels of BMP-2 mRNA in resting zone chondrocytes.

Tritiated thymidine incorporation was used to determine DNA synthesis, and hence, chondrocyte cell proliferation. While the more mature growth zone chondrocytes showed no significant increase in DNA synthesis in response to BMP, the resting zone chondrocytes showed increased tritiated thymidine incorporation at rhBMP-2 levels above 12.5ng/ml. With quiescence, both growth zone and resting zone cells showed increased incorporation. Quiescence is used in an attempt to synchronize the cells; this helps to make the background more consistent. The fact that both cell types showed a response after being placed into quiescence, but not in the non-quiescent groups, suggests one of two possibilities. First, the fact that growth zone cells did not respond in the experiments without quiescence could indicate that the cells have matured, DNA levels are lower, and there is less proliferation. Second, it is possible that without quiescence the increased background might have prevented us from seeing the effect on the growth zone cells, even if it was present. Yet with the results acquired, it appears there is an increase in cell proliferation in the resting zone cells at higher levels of rhBMP-2. This data substantiates findings by Boyan and Schwartz (1991) where it was found that cultured cells became less proliferative and more differentiated as they mature from resting zone to growth zone chondrocytes. Along with the trends found in our alkaline phosphatase experiments, we have confirmed earlier work done by Wuthier, *et al.* (1988), who found that an increase in tritiated thymidine incorporation correlated with concomitant increases in intracellular and matrix vesicle alkaline phosphatase levels.

Collagenase and non-collagenase digestible protein production were assessed by



measuring tritiated proline incorporation. It was found that resting zone cells in response to BMP produced more collagenase digestible protein but less non-collagenase digestible protein than growth zone cells. As with the alkaline phosphatase experiments, the increases for collagenase digestible and non-collagenase digestible protein both occurred in the range of 12.5 ng/ml of rhBMP-2 (rhBMP-2 concentrations of 3.1-25 ng/ml). This result follows what would be expected in endochondral ossification in the growth plate. That is, resting zone cells in the early stages are synthesizing collagenous matrix in the growth plate and as such we would see an increase in collagenase digestible and non-collagenase digestible protein levels. Growth zone cells, on the other hand, would be expected not to produce as much collagenous matrix proteins but perhaps more non-collagenous proteins like alkaline phosphatase as the cells approach the zone of calcification. Yet, with net increases in collagenase digestible and non-collagenase digestible protein levels, the percent collagen production remained unchanged.

Clouding the issue, however, are the results from the tritiated uridine experiments indicating total RNA production. In the uridine experiments, both growth zone and resting zone cells showed a significant increase in protein synthesis at the higher levels of BMP (statistically significant peaks began at 25ng/ml for both cell types). During the time course part of the experiment, though, it appears that growth zone chondrocytes did not respond to BMP after 48 hours. The BMP-2 concentrations which produced significant increases in protein synthesis stimulation were also slightly higher than those concentrations that produced significant increases in the collagenase and non-collagenase digestible protein. The peaks occurred at different rhBMP-2 concentrations; the increase in collagenase/non-collagenase digestible protein synthesis did not correlate with that of increased RNA production. One

reason for this is that the RNA analysis used is not a particularly specific or sensitive assay. We also know that the cartilaginous matrix undergoes continual synthesis and degradation. In this experiment, total protein was measured. Although we know the relative timing in the ossification cascade for the synthesis of some matrix proteins (i.e., alkaline phosphatase production), it is possible the amounts of RNA production were so low compared to the total protein released from cellular synthesis and breakdown, one may not see evidence of the RNA increase; it is possible there was an increase in RNA production correlated with the non-collagenase digestible protein peak synthesis, but was undetectable.

In summary, we have shown that rhBMP-2 regulates chondrocyte proliferation, differentiation, and metabolism, however, this regulation is maturation-dependent. It appears that the release of BMP-2 is autocrine in nature. The results also suggest that new bone formation induced by BMP-2 may occur through direct regulation of chondrocyte differentiation.

To our knowledge, the results presented here are some of the first to show an effect of a purified BMP-2 on chondrocytes. There is a paucity of knowledge as to how chondrocytes are affected by individual pure growth factor matrix proteins (including BMP). There is also a lack of knowledge concerning how these factors interrelate to one another *in vivo*. With these results, several concerns surface. First, there is a need for a greater understanding of the regulation of rhBMP-2 over a course of time. Our results show how growth zone and resting zone chondrocytes respond at 24 and 48 hours. Boyan, *et al.* (1992a) however, showed that it took at least 3 days for increases in hyaluronic acid and 10 days for chondroitin sulfate levels to develop. "More than three days may be required for complete expression of the chondrocyte

phenotype typical of endochondral ossification (Boyan, *et al.*, 1992a)." Second, how the rhBMP-2 concentrations used in these experiments compare to those levels found *in vivo* are not well understood. Extrapolation of our data indicates that a significant increase of activity continue beyond our highest dose levels ( $> 100\text{ng/ml}$ ). Future work should include rhBMP-2 treatment levels greater than  $100\text{ng/ml}$ , as well as studies of the BMP-2 levels commonly found within the different zones found occurring in growth plates.

In dentistry, alveolar bone is the mainstay for a functional dentition. Non-union alveolar fractures, large bone defects from aggressive dysplastic bone lesions, alveolar bone loss from periodontal disease, and atrophic alveolar processes secondary to edentulous jaws (Nilsson, 1985) diminish a patient's ability to chew, speak, or to feel confident about their appearance. Gaining a clear understanding of the mechanisms of ossification could have a wide range of clinical applications that address these concerns.

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## VI. VITA

Douglas M. Erickson was born May 15, 1954, to Donald Milton and Ernestine Held Erickson in Minneapolis, Minnesota. Following graduation from the Wayzata High School in Wayzata, Minnesota, he attended St. Olaf College in Northfield, Minnesota where he earned his Bachelor of Arts degree in biology in 1977. During his time at St. Olaf, he earned a position on the deans list each year, graduating cum laude. Dr. Erickson received his Doctor of Dental Surgery from the University of Minnesota School of Dentistry in 1981. During dental school he earned a position on the deans list all four years. He also earned the Rolland R. Jones award for academic achievement his last 2 years, and in 1981, he was elected to the Omicron Kappa Upsilon National Honor Society and graduated cum laude. Following his dental training at Minnesota, Dr. Erickson completed a general practice residency at the Denver General Hospital, Denver, Colorado in 1982. From 1982-83, he held various associateships in Denver prior to beginning his own general practice in Frisco, Colorado in the fall of 1983. Dr. Erickson was commissioned as a captain in the United States Air Force in 1988 and since has been promoted to the rank of major. After a tour at David Grant USAF Medical Center at Travis AFB, California, he was accepted into the prosthodontic residency at the Wilford Hall Medical Center at Lackland AFB in San Antonio. He anticipates a graduation date of June 1994 at which time his fate will be cast to winds. Dr. Erickson has been married to his wife Nancy for 10 years and together they have a 8 year old daughter, Sarah. Upon completion of training, he anticipates continuing his career and service with the US Air Force.